Binding of Calcium(II) and Lanthanum(III) by a Microbial Ionophore. Evidence for a Dynamic, Intramolecular Donor Exchange in Complexes of Lasalocid A†

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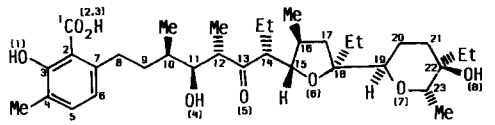
ABSTRACT: Calcium(II) and lanthanum(III) complexes of lasalocid A (X-537A), a carboxylic, polyether antibiotic, have been isolated and investigated in chloroform solution by proton nuclear magnetic resonance (NMR) spectroscopy. The crystalline complexes have the formulation $Ca(LAS)_2 \cdot X CHCl_3$ and $La(LAS)_3 \cdot X CHCl_3$, where $X = 1$ or 2 and LAS is the anion of lasalocid A. Proton NMR spectra of these complexes at ambient temperature contain both narrow and unexpectedly broad signals. Changes in the spectra over the temperature range 240–365 K indicate that the origin of signal broadening is an intramolecular exchange process involving oxygen donors. These data and observed changes in proton NMR signals during titration experiments, in which bound Na^+ is replaced by Ca^{2+} or La^{3+}, are used to infer cation binding sites of the ionophore. A solution structural model is proposed for $Ca(LAS)_2$ in which both ligands are bound to Ca^{2+} via O_3 and O_8, but the ligands take turns binding via O_4, O_6, and O_7. A similar model is proposed for $La(LAS)_3$ with on–off binding by O_4 and O_7, and there is also evidence for an exchange process involving the salicylate moiety.

Lasalocid A (X-537A), I,[1] is a member of a group of mi-



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crobial ionophores known as the polyethers (Westley, 1982; Ovchinnikov, 1979; Poonia & Bajaj, 1979; Pressman, 1976; Westley, 1975; Ovchinnikov et al., 1974). These flexible, open-chain antibiotics function as mobile carriers of cations across natural and artificial membranes. The unique features of lasalocid A are (1) the presence of an aromatic ring and (2) its ability to transport not only monovalent cations but also divalent and trivalent cations, as well as biogenic amine cations.

The ability of the polyethers to influence biological processes dependent upon ion transport (or lack thereof) has led to studies of their effects in diverse areas such as photosynthetic phosphorylation (Pressman, 1976; Ovchinnikov et al., 1974), release of biogenic amine neurotransmiters (Westley, 1975), cardiovascular stimulation (Pressman, 1976; Westley, 1975), and distribution of radionuclides for organ imaging (Ovchinnikov et al., 1974). Lasalocid A has been particularly useful in helping to unravel the mechanisms of calcium-dependent physiological processes (Pressman, 1976; Westley, 1975).

The research described in this paper was prompted by our interest in proteins and other biological molecules that bind or act as channels for calcium. It is interesting to note that sodium ions can often move through calcium channels but that these channels are blocked by both magnesium and lanthanum. We were particularly interested in discovering how an extremely flexible, potentially multidenate ligand such as the lasalocid A anion (hereafter abbreviated LAS) binds calcium in solvents of low polarity and how this binding compares with that of lanthanum. This paper presents the results of a nuclear magnetic resonance (NMR) study of Ca^{2+} and La^{3+} complexes of LAS in chloroform-*d* ($CDCl_3$) solution.

Experimental Procedures

NaLAS was purchased from Aldrich Chemical Co. and used without further purification. Elemental microanalyses indicate no significant amounts of water or other solvent molecules. Anal. Calcd for NaLAS: C, 66.64; H, 8.72. Found: C, 66.41; H, 9.03. Proton and carbon-13 NMR chemical shifts for NaLAS are in good agreement with reported values (Seto et al., 1978; Schmidt et al., 1974), and no NMR signals attributable to decomposition products could be detected.

$Ca(LAS)_2$ was prepared by the following procedure. One gram of NaLAS is dissolved in 25 mL of $CHCl_3$. An aqueous solution containing 2 g of $Ca(NO_3)_2 \cdot 4H_2O$ in 30 mL is divided into three portions. One portion is added to the NaLAS solution, and the mixture is stirred vigorously for 2–3 h. The aqueous layer is removed, and the above procedure is repeated with the second and third portions of $Ca(NO_3)_2$ solution. Afterward, the $CHCl_3$ solution is washed with water and then filtered. $CHCl_3$ is removed from the filtrate in a rotary evaporator cooled with liquid N_2. Elemental analyses of the crystalline, white product indicate the presence of one or two molecules of $CHCl_3$, depending upon the temperature during solvent evaporation. Anal. Calcd for $Ca(LAS)_2 \cdot CHCl_3$: C, 61.89; H, 8.05. Found: C, 62.04; H, 8.01. Anal. Calcd for $Ca(LAS)_2 \cdot 2CHCl_3$: C, 57.65; H, 7.46. Found: C, 57.73; H, 7.35.

An analogous procedure, using $La(NO_3)_3 \cdot 6H_2O$ (Alfa Ultrapure), led to $La(LAS)_3 \cdot CHCl_3$ and $La(LAS)_3 \cdot 2CHCl_3$. 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. The presence of $CHCl_3$ in these complexes was verified by observation of a proton NMR signal at 7.1 ppm for solutions of the complexes in cyclohexane-d_{12}.

Proton NMR spectra were recorded at 300 and at 80 MHz on Bruker WM-300 and WP-80 instruments located at the University of Oxford and at The University of Kansas, respectively. Carbon-13 spectra at 20 MHz were run on the

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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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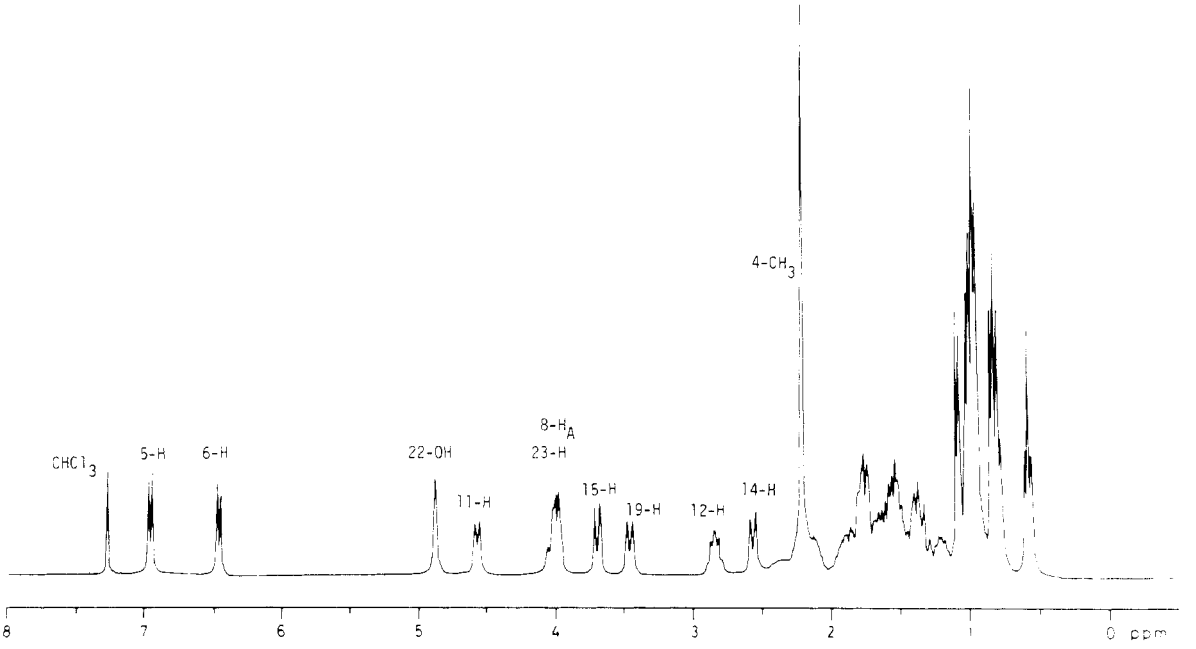

FIGURE 1: 300-MHz proton NMR spectrum of NaLAS in $CDCl_3$ solution at 300 K. Assignments shown are taken from previous studies (Schmidt et al., 1974; Anteunis, 1976; Patel & Shen, 1976a).

WP-80 spectrometer. A few proton spectra were obtained on a home-built, 470-MHz spectrometer at Oxford. Spectra at elevated temperatures were obtained at 80 MHz on samples in sealed tubes. All NMR solutions were freshly prepared.

"Titration" experiments were carried out by adding carefully measured increments of a stock solution of NaLAS in $CDCl_3$ (11.6 mg/mL) to $CDCl_3$ solutions of $Ca(LAS)_2 \cdot CHCl_3$ (1.0 mg/0.5 mL) or $La(LAS)_3 \cdot CHCl_3$ (1.7 mg/0.5 mL) in 5 mm diameter NMR tubes. The solutions were mixed well after each increment. Spectra were recorded at 300 K on both the 300- and the 80-MHz spectrometers with 8K data points and 128 scans.

Results

Proton NMR signal assignments for HLAS and its Li^+, Na^+, Ag^+, and Ba^{2+} complexes have been made in several solvents by double-resonance techniques and by examination of various homologues, isomers, and derivatives (Patel & Shen, 1976a,b; Anteunis, 1976; Schmidt et al., 1974). In chloroform solution, the assignments for NaLAS are in agreement except for the methyl substituents at positions 10, 12, and 16. A typical spectrum of NaLAS in $CDCl_3$ solution at 300 MHz is presented in Figure 1. Signals are generally narrow, and spin coupling is readily observed. Not shown is a broad signal ~14 ppm downfield, which is assigned (Patel & Shen, 1976a) to the hydrogen-bonded salicylate hydroxyl at position 3. The relatively broad signal at 4.85 ppm has been assigned (Anteunis, 1976) to 22-OH on the basis of sharpening of the 23-H quartet upon irradiation of this signal. Analysis of vicinal coupling constants indicates that the Na^+-bound LAS anion in $CDCl_3$ solution adopts very nearly the same cyclic conformation found in X-ray crystal structures of LAS complexes (Patel & Shen, 1976a,b; Anteunis, 1976). This results in an interesting anisotropic effect of the aromatic ring and/or the carboxylate carbonyl on the methylene protons 8-H_A and 8-H_B. Their reported chemical shifts are 3.98 and 2.23 ppm, respectively, for NaLAS in $CDCl_3$ (Anteunis, 1976).

Ca(LAS)$_2$. The 300-MHz proton NMR spectrum of $Ca(LAS)_2 \cdot CHCl_3$ in $CDCl_3$ solution is shown in Figure 2. Signal assignments were made by following the changes in NaLAS signals as the Ca^{2+}/LAS mole ratio was increased during "titration" experiments (vide infra). The most striking feature of the $Ca(LAS)_2$ spectrum is the occurrence of both narrow and broad resonances. Spectra obtained in *N,N*-dimethylformamide and cyclohexane solutions also show this effect. Signals at high field, attributed to methyl and methylene protons, and those at low field, assigned to aromatic protons, are sharp and clearly show spin coupling. On the other hand, signals in the 2.5–5 ppm region, arising from 11-H, 15-H, 19-H, and 23-H, appear considerably broader than those in the spectrum of NaLAS, and in some cases, appreciable changes in chemical shift occur. Significantly, these protons are attached to oxygen-bearing carbons. The signal of 22-OH is not apparent in the spectrum of the calcium complex, but a broad signal assigned to 3-OH appears at 14 ppm. The signal of 14-H is broadened and shifted partially under the envelope of the 12-H signal.

Since the signal broadening observed in the 2.5–5 ppm region of the spectrum of $Ca(LAS)_2$ suggests an equilibrium among species for which chemical shift differences are of the same order of magnitude as the exchange rate, the following experiments were carried out. (1) A proton NMR spectrum in $CDCl_3$ was recorded at 470 MHz to determine whether the increased chemical shift differences would result in effective slow exchange. However, no significant differences in line widths between spectra at 300 and 470 MHz were observed. (2) Proton NMR spectra at 300 MHz in $CDCl_3$ were recorded at lower temperatures in an effort to observe the system under conditions of slow exchange. Even at 240 K, the lowest temperature attained, signals in the 2.5–5 ppm region are considerably broader than those of NaLAS at 300 K. However, in spectra recorded below 265 K, signals assigned to 6-H, 11-H, 14-H, and 19-H appear to split into two components (one of which is several times more intense than the other), and spin splitting is observed in both components of the 6-H and 19-H signals. The results indicate attainment of slow exchange for these four protons. (3) Spectra of $Ca(LAS)_2$ in $CDCl_3$ at elevated temperatures were recorded at 80 MHz, where there is a better chance of attaining fast exchange because of smaller chemical shift differences. Signals in the 2.5–5 ppm region sharpen considerably on going from 300 K to 350 K, and at 365 K, line widths approach those found in

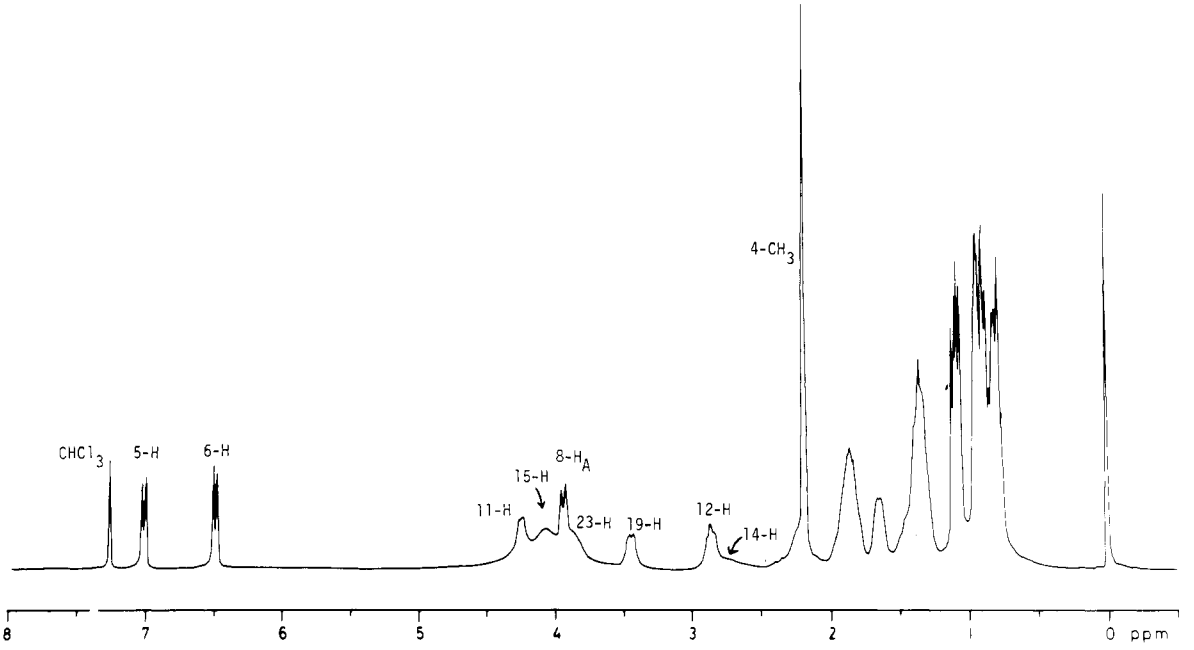

FIGURE 2: 300-MHz proton NMR spectrum of $Ca(LAS)_2$ in $CDCl_3$ solution at 300 K.

the spectrum of NaLAS (see Figure 3). Signal assignments at 365 K were made by following the changes in individual signals at 10 K intervals as the temperature was increased. The observed changes reverse when the temperature is returned to 300 K. Notable changes in individual signals are (see Figure 3) as follows. (a) The 8-H_A signal becomes a doublet of doublets (J = 9.6 and 3.2 Hz) as the temperature is raised. Only the 9.6-Hz coupling is observed at 300 K. (b) The group of resonances attributed to 12-H develops into what appears to be two overlapping quartets as the temperature is raised. (c) The signal assigned (Anteunis, 1976) to 8-H_B, which is largely obscured by the 4-CH_3 resonance at 300 K for both $Ca(LAS)_2$ and NaLAS, moves downfield to ~2.5 ppm as the temperature increases. Very likely, the 14-H resonance, which is very broad and beneath the 12-H signal at 300 K, is part of the envelope of signals centered at ~2.5 ppm at 365 K. Unfortunately, the spectrum at 365 K is not sufficiently well resolved to make more detailed assignments. However, the observed changes in resonances in the 2.5–5 ppm region strongly indicate an approach to fast exchange conditions.

In an effort to identify the oxygen atoms of LAS involved in Ca^{2+} binding in chloroform solution, a NMR titration experiment was carried out as described under Experimental Procedures. A spectrum of the $Ca(LAS)_2$ sample in chloroform solution was recorded at 300 MHz, and then increments of a chloroform solution of NaLAS were added so as to reduce the Ca^{2+}/LAS mole ratio from 0.50 to 0.016 in steps of ~0.03. At a ratio of 0.016, the spectrum is the same as that of NaLAS except for slight broadening of signals in the 2.5–5 ppm region (compare Figures 1 and 4A). Signal assignments for $Ca(LAS)_2$ were made by following changes in individual signals, begining at low Ca^{2+}/LAS where the spectrum matches that of NaLAS.

During the titration, pronounced signal changes occur in the 2.5–5 ppm range. Representative spectra are shown in Figure 4. With increasing Ca^{2+}/LAS, the signal assigned to 22-OH broadens and disappears. The 14-H signal broadens and shifts under the envelope of the 12-H signal. The latter remains largely unchanged, and spin splitting is observed throughout the titration. The other signals in this range undergo significant broadening and, in some cases, chemical shift changes as the Ca^{2+}/LAS ratio is increased. An exception

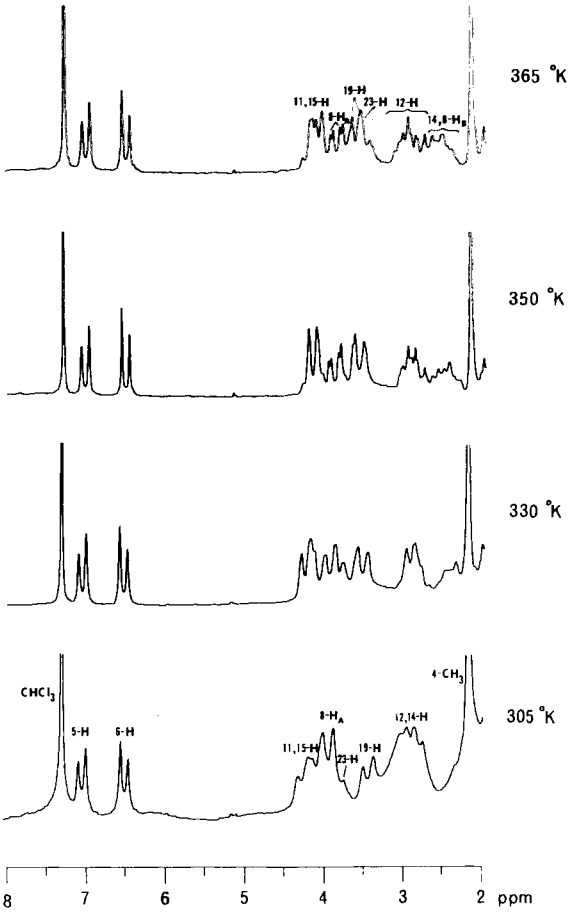

FIGURE 3: 80-MHz proton NMR spectra of $Ca(LAS)_2$ in $CDCl_3$ solution showing effects of increasing temperature on line widths in the 2.5–5 ppm region.

to this may be the 8-H_A signal, which is obscured in most cases by the broad 23-H signal. The largest chemical shift changes are found for 15-H (0.4 ppm), 11-H (0.3 ppm), and 14-H (0.2 ppm). With increasing Ca^{2+}/LAS, the relative order of signal broadening is 22-OH > 11-H, 14-H, 15-H, and 23-H > 19-H > 12-H. A well-isolated triplet at high field (0.58 ppm) in the spectrum of NaLAS, assigned (Anteunis, 1976; Schmidt

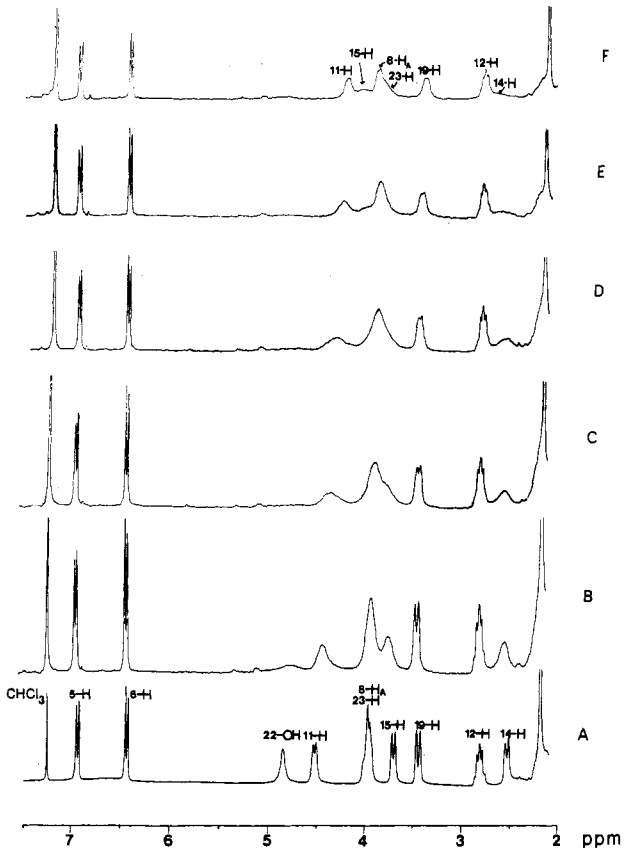

FIGURE 4: 300-MHz proton NMR spectra in $CDCl_3$ solution illustrating various stages in the titration of NaLAS with $Ca(LAS)_2$. Ca^{2+}/LAS mole ratios are (A) 0.016, (B) 0.14, (C) 0.22, (D) 0.31, (E) 0.38, and (F) 0.47.

et al., 1974) to the methyl group of the ethyl substituent at either C_{14} or C_{18}, broadens somewhat and shifts downfield by at least 0.2 ppm as the Ca^{2+}/LAS ratio is increased. At Ca^{2+}/LAS = 0.35, this signal is under the envelope of signals assigned to other methyl groups. The titration experiment was repeated at 80 MHz. Chemical shift changes are the same as those at 300 MHz, but signals in the 2.5–5 ppm region are narrower at 80 MHz, and spin coupling can be observed throughout the titration.

La(LAS)$_3$. The 300-MHz proton NMR spectrum of $La(LAS)_3 \cdot CHCl_3$ in $CDCl_3$, Figure 5, is similar to that of $Ca(LAS)_2$ in that broad resonances are found in the 2.5–5 ppm region for both complexes. Signal assignments were made by following progressive changes during the NaLAS–$La(LAS)_3$ titration described under Experimental Procedures. Notable differences in the spectra of $La(LAS)_3$ and $Ca(LAS)_2$ in the 2.5–5 ppm region are (1) the appearance of a much narrower 14-H signal at 2.65 ppm for the La^{3+} complex,[2] which is close to its position (2.56 ppm) in NaLAS, and (2) a change in the pattern of signals arising from 8-H_A, 15-H, and 23-H. All three are at higher field in the La^{3+} complex, and 15-H has nearly the same chemical shift in the La^{3+} and Na^+ complexes.

Experiments designed to test for the occurrence of exchange processes were carried out for $La(LAS)_3$ in the same manner as described above for $Ca(LAS)_2$. Spectra at 470 MHz proved not to be significantly different in the 2.5–5 ppm region from those obtained at 300 MHz. Low-temperature spectra, obtained at 300 MHz in $CDCl_3$, proved more interesting. At 290 K, signals assigned to 6-H and 4-CH_3 broaden considerably, and at 275 K and below, these signals and the one assigned to 5-H each split into two signals with relative areas of approximately 1:3, thus indicating the attainment of slow exchange. Also, the carbon-13 NMR spectrum of $La(LAS)_3$ in $CDCl_3$ shows distinct splitting of the C_2 signal into two signals of approximate area ratio of 1:3 when the temperature is lowered to 260 K. No other carbon-13 signals appear split at this temperature. There is considerable overlap of proton signals in the 2.5–5 ppm region at 240 K, and it cannot clearly be ascertained whether splitting occurs also for these signals. Spectra at higher temperatures, obtained on the 80-MHz spectrometer, show narrowing of signals in the 2.5–5 ppm region with increasing temperature. At 365 K, signals in this region are as narrow as those of 5-H and 6-H, and complex patterns appear as a result of spin coupling. Signal overlap is greater than that in the Ca^{2+} complex at this temperature, and specific signal assignments for 8-H, 11-H, 15-H, 19-H, and 23-H were not attempted. However, the results indicate fast exchange above 360 K.

A 300-MHz NMR titration experiment in which increments of NaLAS were added to a solution of $La(LAS)_3$ in chloroform was carried out in the same manner as that involving $Ca(LAS)_2$. The La^{3+}/LAS mole ratio was varied from 0.33 to a minimum of 0.029, at which point the spectrum is nearly identical with that of NaLAS (see Figure 6A). With increasing La^{3+}/LAS, significant changes in line widths and, in some cases, chemical shifts occur in the 2.5–5 ppm region, as is the case during the $Ca(LAS)_2$–NaLAS titration. Representative spectra are shown in Figure 6. The relative order of signal broadening is 22-OH > (8 + 23)-H and 11-H > 12-H, 14-H, and 19-H > 15-H. Also, the signals assigned to 11-H, (8 + 23)-H, and 22-OH lose intensity as new signals appear nearby, which gain intensity with increasing La^{3+}/LAS (see arrows in Figure 6). Chemical shifts of the original and new signals, respectively, are (11-H) 4.6 and 4.3 ppm, (22-OH) 4.85 and ~5.4 ppm, and [(8 + 23)-H] 4.0 and ~3.6 ppm. The behavior of the high-field ethyl triplet, assigned (Anteunis, 1976; Schmidt et al., 1974) to the substituent at either C_{14} or C_{18}, parallels that observed during the $Ca(LAS)_2$–NaLAS titration. This signal shifts downfield into the envelope of aliphatic resonances as La^{3+}/LAS increases.

Discussion

X-ray crystallographic studies of the Na^+, Ag^+, and Ba^{2+} complexes of LAS, carried out in the 1970s, show the LAS anion to occur in a cyclic conformation, stabilized by hydrogen bonds between the carboxylate group and O_4-H and O_8-H (Schmidt et al., 1974; Johnson et al., 1970; Maier & Paul, 1971; Chiang & Paul, 1977; Smith et al., 1978). Most oxygen atoms are directed inward, forming a hydrophilic pocket in which the cation resides. The Na^+ and Ag^+ complexes are dimers with two cations sandwiched between two cyclic LAS anions. Cation binding involves O_4, O_5, O_6, O_7, and O_8 in each case. In one of the Na^+ complexes, O_3 is also bound, and Ag^+ is π-bonded to the aromatic ring in the Ag^+ complex. The Ba^{2+} complex is a bis complex in which one anion binds via O_3, O_4, O_5, O_6, O_7, and O_8 while the other anion uses only O_3 and O_8 for cation binding. The peripheral groups in all these complexes are hydrophobic, a fact that has been proposed to explain their high solubility in nonpolar solvents and in nonpolar regions of lipid membranes. This, of course, assumes that gross structural changes do not occur on going to the solution phase.

Numerous attempts to establish the structures of LAS complexes in solution have been made by a variety of spectroscopic techniques (Lallemand & Michon, 1978; Patel &

[2] This is obscured by a water signal in Figure 5, but it is evident in spectra from other samples (see Figure 6F).

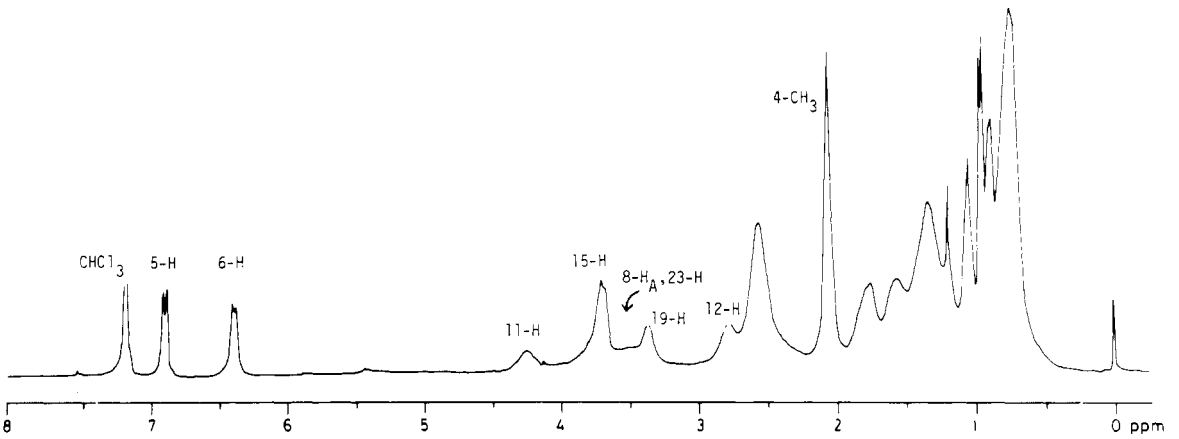

FIGURE 5: 300-MHz proton NMR spectrum of $La(LAS)_3$ in $CDCl_3$ solution at 300 K.

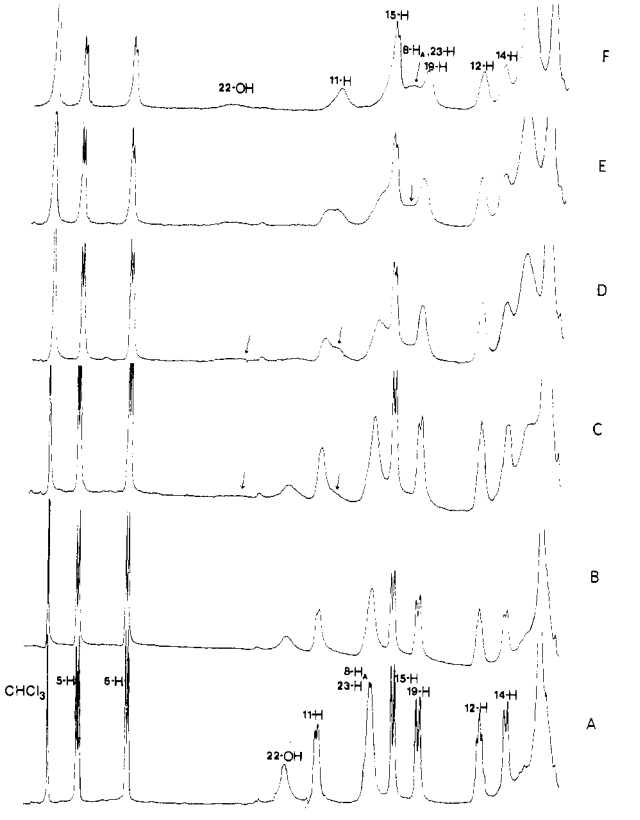

FIGURE 6: 300-MHz proton NMR spectra in $CDCl_3$ solution illustrating various stages in the titration of NaLAS with $La(LAS)_3$. La^{3+}/LAS mole ratios are (A) 0.029, (B) 0.091, (C) 0.133, (D) 0.19, (E) 0.24, and (F) 0.31.

Shen, 1976a,b; Anteunis, 1976; Schmidt et al., 1974; Lallemand et al., 1980; Chen & Springer, 1978; Richardson & Gupta, 1981; Degani & Friedman, 1974a,b, 1975; Briggs et al., 1980; Alpha & Brady, 1973; Krishnan et al., 1978; Painter et al., 1982; Haynes & Pressman, 1974; Hanna et al., 1983). Although in some cases the results appear to indicate the solid-state structures are maintained in the solution phase, there is increasing evidence that structures of LAS complexes in solution depend upon both cation change and solvent polarity.

Perhaps the most definitive data regarding binding by a *monovalent* cation in chloroform solution is that from ^{13}C NMR studies of the Tl^+ complex (Lallemand & Michon, 1978). At low temperatures, spin splitting of LAS ^{13}C signals by the Tl nucleus ($I = {}^1/_2$) is observed for C_1, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{18}, C_{21}, C_{22}, and methyl and methylene substituents assigned to C_{22} and C_{18}, respectively. This is strong evidence for Tl^+ binding by O_3, O_5, O_6, and O_8. The fact that no splitting occurs for C_3, C_{11}, C_{19}, or C_{23} indicates that O_1, O_4, and O_7 are not significantly involved in Tl^+ binding. Evidence that Na^+ also binds to O_5 but probably not to O_4 in solution derives from changes in ^{13}C NMR chemical shifts in CD_2Cl_2 on going from lasalocid acid to NaLAS. A change of 5.8 ppm is observed for C_{13}, whereas the change is only 1.8 ppm for C_{11} (Seto et al., 1978). Furthermore, it has been reported that the ionophoric activity of lasalocid A is not lost upon acetylation at the phenol group, implying that cation binding at O_1 is unimportant (Pressman, 1976).

In a recent ^{13}C NMR study (Hanna et al., 1983), Mn^{2+} was used as a probe of *divalent* cation binding by LAS in both polar and nonpolar solvents. The effects of the paramagnetic Mn^{2+} ion on ^{13}C spin–lattice relaxation rates were used to identify the cation binding sites. In chloroform solution, Mn^{2+} clearly binds to O_3, O_4, O_7, and O_8, but there is no evidence for binding to O_1 and O_5. Weaker binding to O_6 cannot be ruled out. The significant differences between these results and those cited above for Tl^+ concern O_4, O_5, O_7, and possibly O_6. Further support for binding of monovalent but not divalent ions to O_5 in solution is the observation that the affinity of lasalocid A for monovalent ions relative to divalent ions is depressed upon reduction of the C_{13} carbonyl (Pressman, 1976).

Ca(LAS)$_2$. The spectrum of $Ca(LAS)_2$ at 30 K suggests a dynamic process involving LAS substituents near those protons giving rise to broad resonances. Experimental evidence in support of an exchange process occurring at an "intermediate" rate on the proton NMR timescale and affecting NMR signals in the 2.5–5 ppm region is presented under Results. The evidence includes (1) splitting of some signals at low temperature in the 300-MHz spectra, indicating attainment of slow exchange, and (2) a pronounced decrease in NMR line widths (with well-resolved spin coupling) at high temperatures in the 80-MHz spectra, which implies fast exchange on the NMR timescale.

Since the exchange-broadened signals arise from protons near potential cation binding sites, it is reasonable to assume the exchange involves Ca^{2+} binding. In a solvent of low polarity such as chloroform, it is unlikely that a dissociative process such as $Ca(LAS)_2 \rightleftarrows Ca(LAS)^+ + LAS^-$ is significant. It is more likely that an *intra*molecular exchange occurs during which various combinations of LAS oxygens take turns binding to Ca^{2+}. For example, the two LAS ligands, each using a different combination of oxygen donors, might exchange roles so as to maintain a constant coordination number for Ca^{2+}.

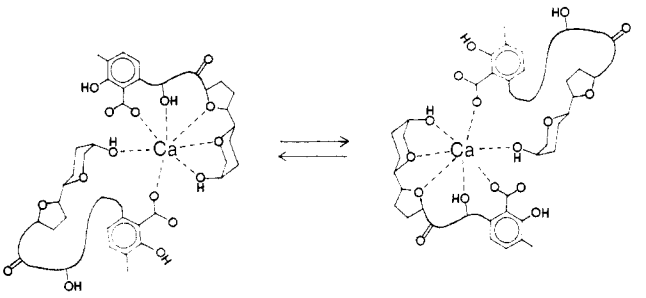

FIGURE 7: Scheme showing intramolecular donor exchange proposed for $Ca(LAS)_2$ in $CDCl_3$ solution.

This is suggested by the structure found for $Ba(LAS)_2$ in the solid state (Johnson et al., 1970), where the two LAS ligands are bound quite differently to Ba^{2+}. The broad signals observed for 11-H, 15-H, 19-H, and 23-H in the spectrum of $Ca(LAS)_2$ indicate on–off binding by O_4, O_6, and O_7. The pronounced broadening of the 14-H signal probably indicates a significant conformation change in LAS concurrent with the exchange process. The magnitude of the effect suggests that 14-H is sufficiently near an aromatic ring in *one* of the conformations to receive an anisotropic shift.

During the titration experiment, Na^+ is replaced by Ca^{2+} as the Ca^{2+}/LAS mole ratio increases. In NMR spectra of mixtures of NaLAS and $Ca(LAS)_2$, no extra signals are observed that would indicate slow intermolecular exchange of LAS. Thus, the spectra at various Ca^{2+}/LAS represent averages of the spectrum of $Ca(LAS)_2$, in which some signals are broadened by *intra*molecular exchange processes, and the spectrum of NaLAS, where these signals are much sharper. Thus, observed proton NMR signal changes during the titration provide further information about Ca^{2+} binding sites.

The increasing NMR signal broadening observed for 11-H, 15-H, 19-H, 23-H, and 22-OH with increasing Ca^{2+}/LAS indicates Ca^{2+} binding at O_4, O_6, O_7, and O_8, respectively. The relatively large chemical shift changes found for 11-H and 15-H during the titration also support binding at O_4, and O_6. The 14-H signal shifts downfield by 0.15 ppm and undergoes pronounced broadening as Ca^{2+}/LAS is increased. As discussed earlier, the unusual behavior of this signal suggests that 14-H may be near an aromatic ring in one conformation of Ca-bound LAS.

The binding sites indicated for Ca^{2+} in this work are consistent with those found for Mn^{2+} by ^{13}C spin–lattice relaxation measurements (Hanna et al., 1983). Binding by a carboxyl oxygen, O_3, was also found for Mn^{2+} and is presumed to occur for Ca^{2+}. There are no protons sufficiently near this group to detect binding in the proton NMR experiments. The evidence is fairly strong for binding of O_6 to Ca^{2+}, whereas the ^{13}C NMR results with Mn^{2+} are ambiguous in this regard.

The results presented here lead us to propose the following structural model for $Ca(LAS)_2$ in chloroform solution (see Figure 7). The two ligands bind Ca^{2+} differently in an instantaneous structure. This structure *may* resemble the solid-state structure of $Ba(LAS)_2$, where one ligand binds Ba^{2+} via several oxygens while the other binds only through O_3 and O_8. For $Ca(LAS)_2$ in chloroform solution at ambient temperature, the ligands exchange roles in a dynamic process that maintains a constant coordination number for the Ca^{2+} ion and that occurs at a rate approximately the same order of magnitude as chemical shift differences (~30 Hz) between the off and on environments. Binding sites involved in the exchange process include O_4, O_6, O_7.

La(LAS)$_3$. Although there are differences between the proton NMR spectra of $La(LAS)_3$ and $Ca(LAS)_2$, both have broad signals in the 2.5–5 ppm region, and the arguments given earlier in support of an intramolecular exchange process for $Ca(LAS)_2$ apply also to $La(LAS)_3$. As in the case of $Ca(LAS)_2$, the rate of this process increases to the fast limit on the NMR timescale for $La(LAS)_3$ at temperatures above 360 K. At temperatures as low as 240 K, no signal splitting indicative of slow exchange is apparent in the 2.5–5 ppm region, perhaps as a result of signal overlap. However, below 260 K, the salicylate "head" of LAS clearly has two slowly exchanging environments with relative populations of approximately 1:3. At ambient temperature, these are in fast exchange. The pronounced broadening observed for signals assigned to 11-H, 19-H, and 23-H implies that O_4 and O_7 are involved in a dynamic on–off binding to the La^{3+} ion similar to that proposed for $Ca(LAS)_2$. The 15-H signal is not so severely broadened as in the Ca^{2+} complex, but this does not preclude O_6 involvement in an on–off binding process since the chemical shift change for 15-H may be smaller in the La^{3+} complex. A particularly striking difference between the spectra of the two complexes is the appearance of a much narrower 14-H signal in $La(LAS)_3$. This could mean that the LAS conformation that places this proton near an aromatic ring, proposed for the Ca^{2+} complex, does not occur in the La^{3+} complex.

Binding sites for La^{3+} are expected to be the same as those for Gd^{3+}. A carbon-13 spin–lattice relaxation study (Hanna et al., 1983) of LAS in the presence of Gd^{3+} clearly indicates binding to O_3, O_4, O_7, and O_8 in chloroform solution. Weak binding at O_1 and O_6 may occur, but O_5 is not involved in binding. These results are similar to those obtained with Mn^{2+} as a paramagnetic binding site probe. Unfortunately, there are no X-ray data available for a LAS complex of a trivalent cation to allow comparison of solution and solid-state structures.

During the titration experiment, two signals each from 11-H, 22-OH, and 23-H are observed, indicating slow *inter*molecular exchange of LAS. These three signals have the largest chemical shift differences between NaLAS and $La(LAS)_3$, indicating binding at O_4, O_7, and O_8. Other signals do not show this doubling because of their smaller chemical shift differences. The observed increases in NMR line widths with increasing La^{3+}/LAS, which are most pronounced for 22-OH, 23-H, 11-H, 12-H, 14-H, and 19-H, also support binding at O_4, O_7, and O_8. As discussed above, the possiblity of binding also at O_6 cannot be excluded. No significant changes in salicylate proton NMR signals occur except for 3-OH, which is ~2 ppm toward higher field in the La^{3+} complex than in the Na^+ and Ca^{2+} complexes.

Thus, we conclude that La^{3+} binds LAS in much the same manner as does Ca^{2+} in chloroform solution. At ambient temperature, an intramolecular process involving O_4 and O_7 (and possibly other oxygens) occurs at a rate intermediate on the NMR timescale, while two environments for the salicylate group are in fast exchange. It cannot be ascertained from these data or from those involving Gd^{3+} whether lanthanide ions bind O_6 in chloroform solution. Binding occurs at O_3 but not at O_5. Very likely, the three ligands in $La(LAS)_3$ are bound differently in an instantaneous structure, and at least two take turns binding via O_4 and O_7. The anionic carboxylate is likely bound in all cases in chloroform solution to prevent charge separation. Steric interactions between three bulky LAS ligands must be severe, and this will determine the coordination number of the La^{3+} ion. One or more ligands are very likely "dangling", i.e., bound only at the carboxylate group. The salicylate groups of some ligands appear to be bound differently than in others. Plausible modes of salicylate coordination

include (1) monodenate carboxylate, (2) bidentate (O_2 and O_3) carboxylate, and (3) a bidentate chelate formed by O_1 and O_2 (or O_3) binding. Examples of (1) and (3) in the solid state have been reported for complexes of salicyclic acid (Downie & Speakman, 1954; Klug et al., 1958; Hanic & Michalov, 1960; Kushi et al., 1970), and (3) has been proposed to explain results of a ^{13}C NMR spin–lattice relaxation study of the Cu^{2+} complex of LAS in chloroform solution (Lallemand et al., 1980).

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