

Hydrogen-1 and Carbon-13 Magnetic Resonance Studies of Nonactin–Calcium Complex[†]

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ABSTRACT: For an understanding of the cation selectivity and general binding characteristics of macrotetralide antibiotic nonactin (NA) with ions of different sizes and charges, the nature of binding of divalent cation, Ca^{2+} , to NA and conformation of the NA– Ca^{2+} complex have been studied by use of 270-MHz proton nuclear magnetic resonance (^1H NMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR). The calcium ion induced significantly large changes in chemical shifts for H_7 , H_2 , H_3 , and H_5 protons of NA and relatively small changes for H_{18} and H_{21} protons. Changes in ^{13}C chemical shift were quite large for carbonyl carbon, C_1 ; it is noteworthy that in the NA– K^+ complex, H_2 and H_{21} protons practically do not show any change during complexation and carbonyl carbon shows a much smaller chemical shift change.

It is well-known that the ionic permeabilities in biological membranes are enhanced by certain macrocyclic antibiotics such as valinomycin, enniatin, nactins, etc. (Ovchinnikov et al., 1974; Pressman, 1976; Muller & Rudin, 1967). Nonactin (NA) (Figure 1) is one of the members of macrotetralide nactin group of neutral ionophores and has been shown to enhance K^+ transport to a significantly greater extent compared to other monovalent cations such as Na^+ (Mueller & Rudin, 1967). ^1H NMR studies of NA, NA– K^+ complex, and NA– Na^+ complex have been reported (Prestegard & Chan, 1969, 1970). ^{13}C NMR studies of NA and NA– K^+ have also been reported (Pretsch et al., 1972). It has been shown that NA forms equimolar complexes with K^+ and Na^+ , with almost identical conformation for the complexes.

The stabilities of the Na^+ and K^+ complexes with NA are of the same order in solvents like dry acetone while it gets substantially reduced in the presence of small amounts of water in the case of NA– Na^+ complex. The X-ray crystallographic studies of K^+ and Na^+ complexes of NA (Kilbourn et al., 1967; Dobler & Phizackerley, 1974) have shown that the complexes are by and large isomorphous and have conformations similar to those proposed in solution by using ^1H NMR. While many theoretical models have been proposed to explain the structure and function of many of these antibiotics, substantial experimental evidence to understand the mechanism of ion-transport enhancement and cation selectivity are not available. In order to get a clearer picture on the cation selectivity of NA and its general binding characteristics, it is important to study complexing properties of ions of different sizes and charges. Keeping this in view, the study of complexation of NA with a biologically important divalent alkaline earth metal ion, namely Ca^{2+} , was undertaken. In this paper we report our findings based on ^1H NMR and ^{13}C NMR on the confor-

From an analysis of calcium ion titration data, it is concluded that NA forms an equimolar complex with Ca^{2+} . The study of complexation of NA in dry acetone- d_6 and in acetone- d_6 containing 0.1 mol fraction of D_2O indicated that only unhydrated Ca^{2+} is bound to the NA, similar to complexes of NA with other cations. The binding constant for NA– Ca^{2+} in dry acetone is of the order of 10^4 (in mole fraction units); this gets marginally reduced to 7.5×10^3 in the case of wet acetone. The study further indicated that the overall conformation for NA– Ca^{2+} is similar to that of NA– K^+ reported earlier, with significant local conformational changes occurring in order to accommodate the doubly charged, smaller sized calcium ion.

mation and ion-binding properties of NA with Ca^{2+} .

Experimental Procedures

NA was obtained from Sigma Chemical Co. and was used without further purification. The calcium perchlorate ($\text{Ca}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$), obtained from Alfa Laboratories, was dried over P_2O_5 for several hours before use; 99.8% deuterated acetone and D_2O , obtained from Stohler isotopes, were used as solvents. The ^1H NMR (270 MHz) and proton-noise-decoupled ^{13}C NMR (67.89 MHz) spectra were recorded by using a Bruker WH-270 FTNMR spectrometer equipped with variable-temperature accessories. The spectrometer was field frequency locked by using the deuterium signal of the solvents and was operated at 20 °C for ^1H NMR and at 32 °C for ^{13}C NMR, unless otherwise stated. The reported chemical shifts are in ppm with respect to internal reference Me_4Si . To increase the resolution, in ^1H NMR, the spectra were recorded with a sweep width of 2000 Hz covering a range of δ 0–6 only. The typical number of scans were about 100 and 1000 for ^1H and ^{13}C NMR studies, respectively.

For ^1H NMR studies, the concentrations of NA solution were of the order of 15–20 mM. For ^{13}C NMR studies, the typical concentration of NA was ≈ 35 mM. NA solution was prepared in dry acetone- d_6 (containing minimal water). The solution, for titration studies, was divided into two parts, A and B; to one part (B) calcium salt was added in excess so that the resulting solution contained NA and Ca^{2+} in the ratio of $\approx 1:20$. The ^1H NMR spectrum was recorded for this extreme concentration ratio. To the solution containing free NA (A), solution B was gradually added and spectra were recorded for different molar ratios of Ca^{2+} to NA until further addition of solution of B did not produce any change in the chemical shift values. The final spectrum, within the allowable experimental error limits, was identical with that of the extreme concentration. A similar procedure was adopted for ^{13}C NMR titration as well.

For ^1H NMR studies in solvent mixture (acetone- d_6 + D_2O), a solution of NA in dry acetone was prepared to which calculated quantity of D_2O was added so that the D_2O in the solution mixture was ≈ 0.1 mol fraction (the spectrum re-

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Table I: ^1H NMR Chemical Shifts of NA, NA-K^+ , and NA-Ca^{2+} in Dry Acetone- d_6 and of NA-Ca^{2+} in Acetone- D_2O

sample	solvent	chemical shift ^a (δ)							
		H_7	H_3	H_5	H_2	H_6	$\text{H}_{6'}$	H_{21}	H_{18}
NA	acetone- d_6	4.96	3.99	3.87	2.49	1.74	1.74	1.22	1.05
NA-K^+	acetone- d_6	5.52	4.47	4.14	2.50	1.6–1.8		1.22	1.15
NA-Ca^{2+}	acetone- d_6	5.54	4.27	4.16	2.95	2.00	1.60	1.36	1.25
NA-Ca^{2+}	acetone- d_6 + D_2O (0.1 mol fraction)	5.44	4.20	4.09	2.91	1.94	1.65	1.33	1.23

^a Me_4Si internal standard.Table II: Coupling Constant of NA, NA-K^+ , and NA-Ca^{2+} in Acetone- d_6 and of NA-Ca^{2+} in Acetone- d_6 - D_2O

sample	solvent	coupling constant (Hz)									
		3J ($\text{H}_7, \text{H}_{21}$)	3J ($\text{H}_2, \text{H}_{18}$)	3J (H_3, H_2)	2J ($\text{H}_6, \text{H}_{6'}$)	3J (H_6, H_6)	3J ($\text{H}_5, \text{H}_{6'}$)	3J (H_7, H_6)	3J ($\text{H}_7, \text{H}_{6'}$)	3J ($\text{H}_7, \text{H}_6 + \text{H}_5, \text{H}_{6'}$)	3J ($\text{H}_5, \text{H}_{6'}$)
NA	acetone- d_6	6.3	7.1	7.6	11.7					13.0	12.4
NA-K^+	acetone- d_6	6.3	7.3	9.4						11.1	13.3
NA-Ca^{2+}	acetone- d_6	6.4	7.5	10.0	14.5	10.8	2.7	2.0	10.9	12.9	13.5
NA-Ca^{2+}	acetone- d_6 + D_2O (0.1 mol fraction units)	6.3	7.6	9.8	13.9	10.6	2.7	2.0	11.2	12.0	13.3

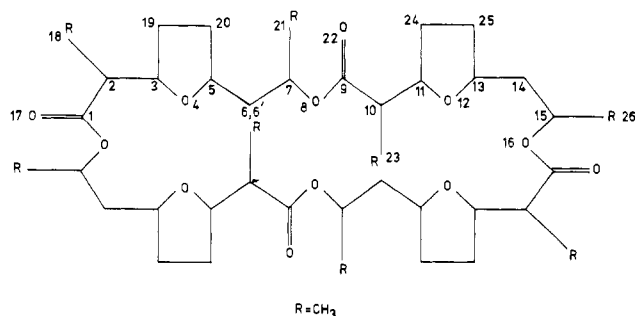


FIGURE 1: Structure of nonactin.

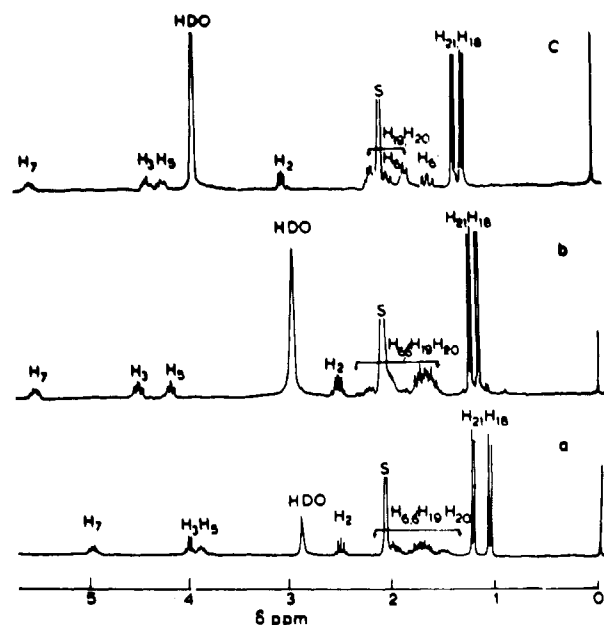
corded for free NA in the solvent mixture containing similar amounts of D_2O was identical with that of free NA in dry acetone- d_6 . Calcium ion titrations were repeated as detailed above until a concentration ratio of 1:4 was reached. To this, D_2O was added in steps of ≈ 0.01 mol fractions, and spectral changes were followed. The changes were gradual, and around 0.18 mol fraction of D_2O , the spectrum resembled that of free NA in acetone.

Results

The 270-MHz ^1H NMR spectra of free NA and its Ca^{2+} complex in dry acetone- d_6 are shown in Figure 2. The spectrum NA-K^+ in dry acetone- d_6 is also included in the figure for comparison. The assignments of various ^1H signals were based on extensive spin-decoupling experiments, and they agree with the assignments reported earlier (Prestegard & Chan, 1969, 1970).

The chemical shifts of various proton signals for free NA, NA-K^+ , NA-Ca^{2+} in dry acetone, and NA-Ca^{2+} in acetone containing 0.1 mol fraction of D_2O (wet acetone) are given in Table I. No significant changes were observed in the signal positions over a temperature range of -30 to $+40$ $^{\circ}\text{C}$; only one set of sharp signals for four subunits of NA was observed for the entire range of concentration studied. Table II gives the coupling constants of the coupled protons of the NA, NA-K^+ , NA-Ca^{2+} in dry acetone- d_6 , and NA-Ca^{2+} in wet acetone.

The proton chemical shifts and the coupling constants reported here for NA-Ca^{2+} were either directly measured (where there was no ambiguity) or were estimated by spin analysis using LACON program followed by a spectral simulation using

FIGURE 2: 270-MHz proton NMR spectra of (a) free NA, (b) NA-K^+ , and (c) NA-Ca^{2+} in dry acetone- d_6 . NA concentration = 18 mM; sweep width 2000; temp 25 $^{\circ}\text{C}$.

ITERCAL. The H_2 signal was analyzed as the X part of P_3XA ($\text{CH}_3(18)\text{-CH}(2)\text{-CH}(3)$) and simulated with the spectrum obtained by irradiation of the $\text{H}_{19}\text{-H}_{19'}$ region. The H_7 signal was analyzed as the X part of ABXP_3 ($\text{CH}(6)\text{-CH}(6')\text{-CH}(7)\text{-CH}_3(21)$) and signal simulation for the H_7 and $\text{H}_6\text{-H}_{6'}$ region was effected with respect to the H_5 proton-decoupled spectrum. The simulated spectral regions of H_2 , H_7 , and $\text{H-H}_{6'}$ protons are shown in Figure 3a-c.

Figure 4a,b gives the Ca^{2+} titration curves for various resonance lines in dry acetone- d_6 and acetone- d_6 + D_2O systems. For both systems, it can be seen that the signals of protons H_7 and H_2 have the largest changes in chemical shift, the difference $\Delta\delta_{\text{max}}$ in chemical shifts between free NA and the fully complexes NA-Ca^{2+} , in dry acetone, for H_7 and H_2 being respectively 0.57 and 0.46 ppm. These limiting shifts get marginally reduced for the acetone containing 0.1 mol fraction of D_2O . In wet acetone system they are 0.48 and 0.42 ppm, respectively. The $\Delta\delta$ value for H_7 is of the same mag-

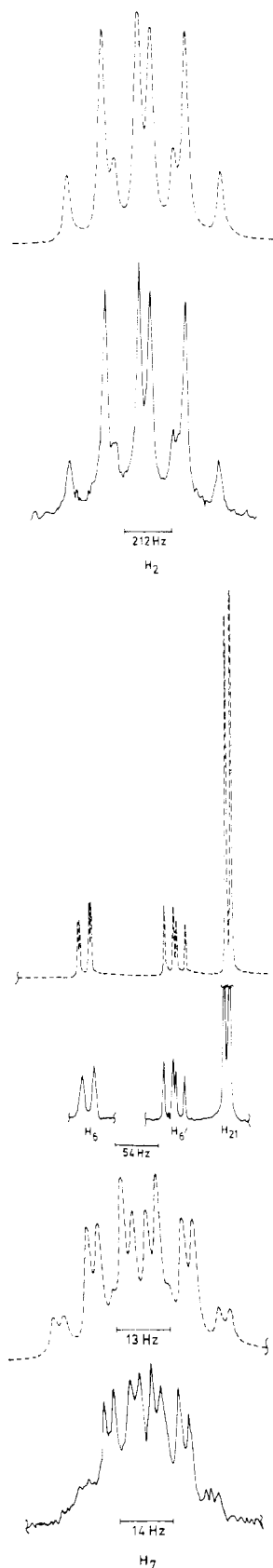


FIGURE 3: Computer-simulated spectra (---) of (a, top) H_2 , (b, middle) H_6 , H_6' , and (c, bottom) H_7 proton along with their experimental spectra (—).

nitude as in the case of $NA-K^+$ while that for H_2 is very much greater in the case of $NA-Ca^{2+}$. $\Delta\delta$ values for signals of H_3 and H_5 are of the same magnitude for $NA-Ca^{2+}$ and $NA-K^+$ in dry acetone. One can also see that, during complexation, the H_6 signal moves downfield by about 0.26 ppm while that

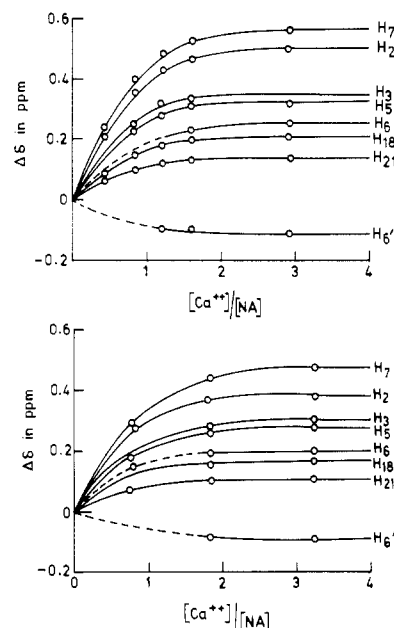


FIGURE 4: Ca^{2+} ion titration curves for proton signals. (a, top) In dry acetone; NA concentration ≈ 18 mM (1.4×10^{-3} mol fraction). (b, bottom) In acetone containing 0.1 mol fraction of D_2O ; Na concentration ≈ 17 mM (1.3×10^{-3} mol fraction).

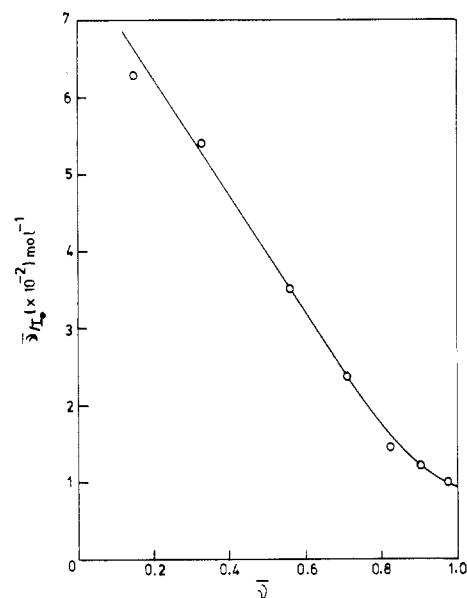


FIGURE 5: Scatchard plot for H_7 proton signal in dry acetone- d_6 . x axis: $\bar{v} = [NA]/[Ca^{2+}] \times \Delta\delta/\Delta\delta_{\max}$. y axis: $\bar{v}/I_0 = \Delta\delta/(\Delta\delta_{\max} - \Delta\delta)C_0$.

of H_6' moves upfield by 0.14 ppm, thus enabling the easy characterization of the otherwise complicated region of methylene protons.

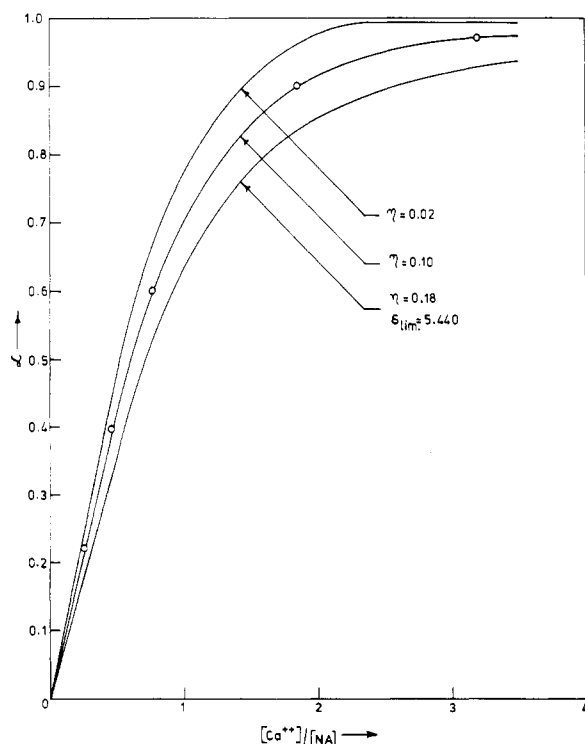
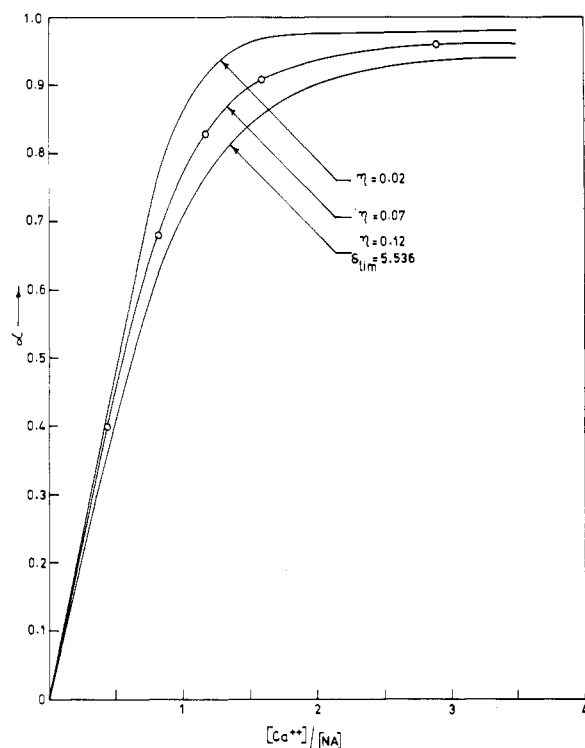
Figure 5 is the Scatchard plot obtained by using the experimental chemical shifts for H_7 and the relation

$$\frac{\bar{v}}{I_0} = nk_f - \bar{v}k_f \quad (1)$$

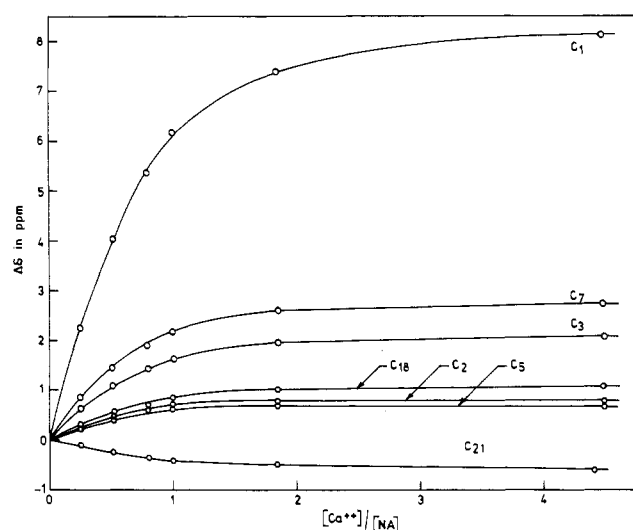
(Reuben, 1973), where \bar{v} is the molar concentration ratio $[NA]/[Ca^{2+}] \times (\Delta\delta/\Delta\delta_{\max})$ and $\bar{v}/I_0 = \Delta\delta/(\Delta\delta_{\max} - \Delta\delta)C$, C being the concentration of the cation, I_0 the initial concentration of the ionophore, and n the stoichiometry of the complex. The slope and intercept obtained from the graph of \bar{v}/I_0 vs. \bar{v} are -742.35 ± 13.75 and 764.5 ± 13.75 , respectively. By use of eq 1, the binding constant K_f and the stoichiometry of the complex, n , estimated are $\approx 740 \text{ M}^{-1}$ and 1.

Table III: ^{13}C NMR Chemical Shifts for NA, NA- K^+ , and NA- Ca^{2+} in Acetone- d_6

sample	chemical shift ^a (δ)								
	C ₁	C ₃	C ₅	C ₇	C ₂	C ₆	C ₂₀	C ₂₁	C ₁₈
NA	174.35	81.16	76.94	69.53	46.00	43.37	32.24	20.92	13.17
NA-K ⁺	177.60	82.34	75.92	68.57	46.56	44.39	32.09	21.02	14.51
NA-Ca ²⁺	182.81	83.36	77.63	72.36	46.81	44.18	32.33	20.37	14.35

^a Me_4Si internal standard.FIGURE 6: Theoretically computed curves of degree of complexation, α , vs. molar concentration ratio for different values of η : (a, top) in acetone- d_6 ; (b, bottom) in acetone- $d_6 + \text{D}_2\text{O}$ (0.1 mol fractions). Experimental points are marked (O).

The theoretically computed (see discussion below) curves of the degree of complexation, α , vs. the molar concentration

FIGURE 7: Ca^{2+} ion titration curves for ^{13}C signals (in dry acetone).

ratio $[\text{Ca}^{2+}]/[\text{NA}]$ for dry and wet acetone systems respectively, are given in Figure 6a,b. The best fit for the dry acetone system is obtained for $\eta = 0.068$ and $\delta_{\text{max}} = 5.536$, from which binding constant K_{dry} comes out to be $\approx 800 \text{ M}^{-1}$. The corresponding η , δ_{max} , and K_{wet} for the wet acetone system are 0.10, 5.44, and $\approx 580 \text{ M}^{-1}$, respectively. Expressed in mole fraction units, the values of K_{dry} and K_{wet} obtained are 1.0×10^4 and 7.5×10^3 , respectively.

The assignments of ^{13}C signals were based on the assignments of signals for free NA in CDCl_3 (Pretsch et al., 1972), and the changes in the signals due to change in the solvent system from CDCl_3 to acetone- d_6 could be easily followed. Table III gives the limiting ^{13}C chemical shifts of various signals for free NA, NA- K^+ , and NA- Ca^{2+} in dry acetone- d_6 . Figure 7 shows the Ca^{2+} titration graph for various ^{13}C signals. $\Delta\eta_{\text{max}}$ for $^{13}\text{C}_1$ carbonyl carbon is significantly larger for NA- Ca^{2+} as compared to that for NA- K^+ . $\Delta\delta$ for $^{13}\text{C}_1$ in the case of the calcium complex is $\approx 8.5 \text{ ppm}$ as opposed to $\approx 3.25 \text{ ppm}$ for the potassium complex.

Discussion

Both ^1H NMR and ^{13}C NMR of NA- Ca^{2+} complex show only one set of signals for the four subunits of NA, indicating that it retains its C_2 symmetry (near S_4) as in the case of free NA and its various complexes with K^+ , Na^+ , and Cs^+ (Prestegard & Chan, 1969, 1970; Pretsch et al., 1972). Also, the sharply defined signals over a wide range of temperature and for the entire range of concentration studied point out that Ca^{2+} in the complex exchanges at a faster rate, on the NMR time scale, between the free and the complexes states.

Figure 6a,b shows that the type of equilibrium for such a rapid state of exchange could be well represented by an equation of the form



The slightly concave curve of the Scatchard plot with an inflection point indicates the possibility of existence of another

species, I_2C , in equilibrium with IC . This could be represented by an equilibrium expression such as



However, the Scatchard plot also indicates that I_2C is very small in proportion to IC and is present only at low concentrations of salt. As the concentration of the salt is increased, more and more calcium ions would be available for binding, and hence at high salt concentrations eq 2 could be taken to be the only equilibrium reaction, to first order of approximation.

With this approximation, the overall equilibrium constant K_1 could be obtained from

$$K_1 = \frac{[IC]}{[I][C]} = \frac{\alpha}{(I_0 - \alpha)(C_0 - \alpha)} \quad (4)$$

where $[IC]$, $[I]$, and $[C]$ are the molar concentrations of the complex, ionophore (NA), and cation (Ca^{2+}); I_0 and C_0 are the initial concentrations of the ionophore and the cation.

Solving (4) for α , we get

$$\alpha = \frac{((I_0 + C_0 + 1/K) - [(I_0 + C_0 + 1/K)^2 - 4I_0C_0]^{1/2})/2I_0}{2\alpha = (1 + \phi + \eta) - [(1 + \phi + \eta) - 4\phi]^{1/2}} \quad (5)$$

where $\phi = C/I$, $\eta = 1/KC_0$, and $\alpha = (\delta_{\text{obsd}} - \delta_{\text{free}})/(\delta_{\text{com}} - \delta_{\text{free}})$. Equation 5 was solved iteratively over η and δ_{com} (as the limiting value observed could be only approximate) for best fit. The best fit was obtained by minimizing $\sum_i |(\alpha_{\text{exptl}} - \alpha_{\text{calcd},i})|$ summed over the concentrations studied (Luigi et al., 1980). From the best fit value of η , K was calculated.

The observed experimental facts, namely (i) limiting shifts for $Na-Ca^{2+}$ in dry and wet acetone are nearly same and (ii) the ratio of the ratio of the salt-induced chemical shifts of any two signals such as H_2 and H_7 for dry acetone to wet acetone, i.e., $(H_2/H_7)_{\text{dry}}/(H_2/H_7)_{\text{wet}}$, is nearly 1 both indicate that Ca^{2+} forms similar 1:1 complexes in both dry and wet acetone systems. As no significant changes in the coupling constants are noticed for the coupled protons in the two systems, the backbone conformations for the calcium complex in both systems are likely to be the same. This is in agreement with the earlier prediction that only nonhydrated cations are bound to NA when complexed. The apparent reduction in the binding constant for the wet acetone system once again points out that the complexing ability of NA with calcium decreases in the presence of D_2O as calcium has greater affinity for solvation than for complexation with NA. The extent of competition between ion solvation and ion complexation determines the apparent binding constant, the reduction in value being accountable in terms of energy spent in desolvating the ion before complexation.

The observed changes in the chemical shifts and coupling constants when salt is added to free NA in solution are to be accounted to terms of various factors such as (i) the conformational rearrangements in the backbone taking place in the flexible nonactin ring while the cation in the central cavity is being accommodated, (ii) changes in the electronic state of the liganding groups binding the cation, (iii) changes in the electron density around other groups induced by divalent calcium. The protons H_7 , H_3 , and H_5 closer to the carbonyl oxygen and the tetrahydrofuran ring oxygen, which are known to be involved in ligand binding in $Na-K^+$, Na^+ complexes, show similar shift for $Na-Ca^{2+}$ as well, indicating thereby the liganding groups are the same, viz., carbonyl oxygens and tetrahydrofuran ring oxygen, even in $Na-Ca^{2+}$. This fact is

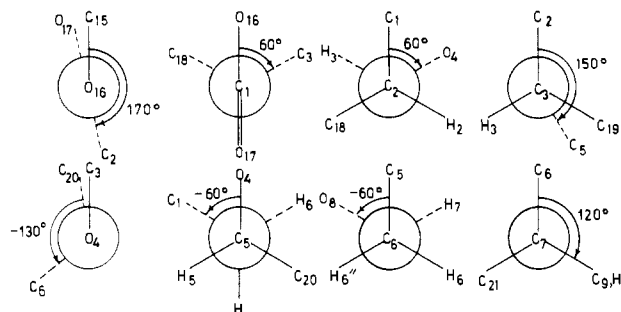


FIGURE 8: Projection diagrams depicting the backbone dihedral angles of $NA-Ca^{2+}$ complex.

corroborated by significant changes in the ^{13}C NMR shifts of C_1 , C_3 , and C_5 carbons.

The significantly large chemical shift change for H_2 noticed in the $NA-Ca^{2+}$ complex alone and the very large ^{13}C NMR chemical shift of carbonyl carbon ($\Delta\delta = 8.2$ ppm) point to the fact that quite a considerable amount of local rearrangement occurs in the backbone around C_1-C_2 . It is reasonable to assume that the four carbonyls orient more steeply toward the cavity and the carbonyl oxygen to cation distance is shorter than the corresponding values in other complexes. The orientation of C_2-H_2 is so changed that the H_2 proton comes, clearly, into the direct influence of the cation apart from the influences due to nearby carbonyl oxygen and ring oxygen. This reorientation is such that the dihedral angle $H_2-C_2-C_1-O$ is nearly zero while $C_1=O$ points directly to the center of the cavity where the cation is held by ion-dipole interactions. In this orientation, the electric field of the cation deshields the H_2 proton, and this deshielding effect is much larger than the possible shielding effect due to anisotropy of $C=O$ nearly syncoplanar with C_2-H_2 . Also, a much smaller deshielding influence on H_2 comes from the nearby ring oxygen. Thus, there is a net downfield shift for the H_2 proton in the $NA-Ca^{2+}$ complex.

The application of a simple Karplus relation for estimating dihedral angles for the vicinal protons shows that H_2 and H_3 are trans, as in the case of $NA-K^+$; H_5-H_6 and H_7-H_6' are also trans while H_5-H_6' and H_7-H_6 are nearly gauche with respect to each other. As the vicinal couplings H_7-H_{21} and H_2-H_{18} do not vary significantly between free NA and $NA-Ca^{2+}$, the methyl groups still point outward from molecule, thus providing a hydrophobic exterior for the complex. The larger magnetic nonequivalence of H_6 and H_6' along with a small, but significant, change in the backbone dihedral angles around $H_5-H_{6,6'}$, $H_{6,6'}-H_7$ account for the spectral simplification of the $H_{6,6'}$ region. The "gauche-trans" and "trans-gauche" orientations of H_7 and H_5 with H_6 and H_6' with near equal coupling constant values for coupling proton pairs with similar relative orientation account for the near-symmetrical sets of signals for H_6 and H_6' protons.

When the above facts in respect to coupling constants and chemical shift changes are taken into account, a model for $NA-Ca^{2+}$ was constructed with the dihedral angles shown in the projection diagrams (Figure 8). Backbone dihedral angles around the bonds about which minimal differences between $NA-K^+$ and $NA-Ca^{2+}$ were observed are taken to be the same as of $NA-K^+$ (Dobler et al., 1969).

The model proposed for the $NA-Ca^{2+}$ complex is shown in Figure 9 and its schematic diagram is given in Figure 10. It is interesting to note that the backbone of both $NA-K^+$ and $NA-Ca^{2+}$ complexes resemble the seam of a tennis ball while the liganding oxygens have different positioning with respect to the "seam". The four tetrahydrofuran ring oxygens are at

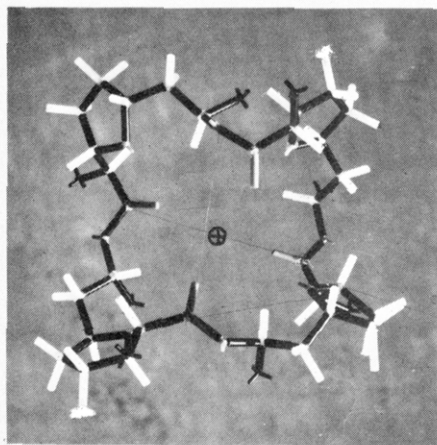


FIGURE 9: Proposed molecular model of NA- Ca^{2+} complex (top view).

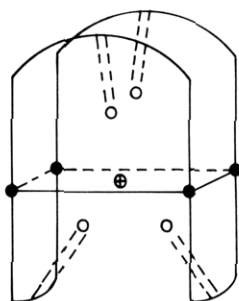


FIGURE 10: Schematic diagram of NA- Ca^{2+} complex.

the vertices of what can be called as an equatorial plane containing the cation at the center while the four carbonyl oxygens, a pair each from the approximate top and approximate bottom of the seam, tetrahedrally coordinate with the central cation. This is to be compared with 8-coordination of NA- K^+ where K^+ ion is at the center of two approximate tetrahedrons formed by the sets of carbonyl and tetrahydro-

furan ring oxygens (Pretsch et al., 1972). The observed opposite effects in the ^{13}C chemical shift changes of the C-5 and C-7 carbon in NA- K^+ and NA- Ca^{2+} are the result of the above-mentioned dissimilarity in local arrangements around the liganding carbonyl and tetrahydrofuran ring oxygens.

The above observations clearly suggest that NA forms almost similar complexes with all of the cations, monovalent and divalent, studied so far. In all cases, it is only the unhydrated cation that is bound to the ionophore, the extent of binding in wet solvents being determined by the relative tendency of complexation and ion desolvation. The small changes noticed in individual complexes could be accounted for in terms of small local rearrangements, the extent of rearrangement being dependent on the size and charge of the cation under consideration.

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