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Circular Dichroism and Nuclear Magnetic Resonance Studies on the Complexation of Valinomycin with Calcium†

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ABSTRACT: Complexation of valinomycin (VM) with the divalent cation Ca^{2+} in a lipophilic solvent, acetonitrile (CH_3CN), has been studied by using circular dichroism and proton and carbon-13 nuclear magnetic resonance (^1H NMR and ^{13}C NMR). From analyses of the spectral data, it is concluded that VM forms a 2:1 (peptide–ion–peptide) sandwich complex with Ca^{2+}, at low concentration of VM. At moderate concentrations of the salt, in addition to the sandwich complex, an equimolar (1:1) complex different from those observed for potassium and sodium is also observed. At very large concentrations of the calcium salt, the data suggested a complex with a conformation similar to that of the free VM in polar solvents. Possible conformations for the sandwich and the equimolar VM–calcium complexes are proposed.

Our understanding of the molecular basis of ion transport across biological membranes is far from complete. However, over the last 2 decades, considerable work has been carried out in different laboratories on the structure and the conformation of ion-binding macrocyclic antibiotics (ionophores) and their cation complexes (Ovchinnikov et al., 1974; Ovchinnikov, 1979; Ovchinnikov & Ivanov, 1974) with a hope to get more insight into their transmembrane ion-transporting properties. Among the ionophores acting as ion carriers, valinomycin (VM), a 36-membered cyclic depsipeptide, *cyclo*-(L-Val-D-Hyi-D-Val-L-Lac)$_3$,[1] plays an important role in selective enhancement of permeability of biological membranes for potassium ions (Pressman, 1968; Tosteson et al., 1967). The circular dichroism (CD) and nuclear magnetic resonance (NMR) studies on VM and its cation complexes have shown that the selectivity of a particular cation by this molecule depends on the nature of the ligands and the conformational states of the molecule (Haynes, 1969; Ivanov et al., 1969; Ohinishi & Urry, 1969; Grell et al., 1973; Patel & Tonelli, 1973; Davis & Tosteson, 1975). The conformation of the free VM has been shown to be highly solvent dependent (Patel & Tonelli, 1973; Ovchinnikov, 1974; Bystrov et al., 1977). A host of literature is available regarding the conformational studies of the complexation of VM with monovalent cations like K^+, Na^+, Rb^+, Cs^+, and Tl^+ (Bystrov et al., 1977; Ovchinnikov, 1974; Davis & Tosteson, 1975; Neupart-Laves & Dobler, 1975; Ohinishi & Urry, 1969). To get more insight into the molecular basis of the ion-transporting properties, it is important also to study the complexing ability of VM with

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[1] Abbreviations: Val, valine; Hyi, hydroxyisovaleric acid; Lac, lactic acid.

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cations of various sizes and charges and in different solvent systems. The studies on the possible conformations that the VM molecule can assume under these varied conditions may be expected to throw light on our understanding of the carrier-mediated ion-transport mechanism. In this paper, we report our results on the conformational studies of VM and its complex with the biologically important divalent cation Ca^{2+}, using CD (in acetonitrile, CH_3CN), ^1H NMR, and ^{13}C NMR (in CD_3CN) techniques.

Experimental Procedures

VM was obtained from Sigma Chemical Co. and was used without further purification. Calcium perchlorate [$Ca(ClO_4)_2 \cdot 6H_2O$] and calcium thiocyanate [$Ca(SCN)_2 \cdot 4H_2O$], obtained through Alfa Laboratories, were vacuum dried over P_2O_5 for several hours before use. Deuterated solvents CD_3CN, $CDCl_3$, and D_2O were obtained from Stohler Isotopes. CD_3CN was preserved over molecular sieves. The CD spectra were recorded in an automatic JASCO-J-20 spectropolarimeter with optical cells of path lengths ranging from 0.1 to 5 mm. The CD instrument was calibrated with 0.01% aqueous *d*-camphor-10-sulfonic acid as the standard. The concentrations of VM solution used for the CD experiments ranged from 1.2 to 15 mM. The reported CD spectra are the mean of two to three spectra on an average. The ^1H NMR (270 MHz) and the proton noise decoupled ^{13}C NMR (67.89 MHz) spectra were recorded on a Bruker WH-270 FT NMR equipped with variable-temperature accessories, at Bangalore NMR Facility, Bangalore, India. The deuterium signal of the solvent was used for the field-frequency locking, and tetramethylsilane (Me_4Si) was used as the internal standard. Typically, about 100 and 1000 scans were used respectively for recording the ^1H NMR and ^{13}C NMR spectra. The concentration of VM solution in the ^1H NMR and ^{13}C NMR experiments was in the range of 5–12 and 25–40 mM, respectively. For the ^1H NMR and CD titration experiments, a single stock solution of known VM concentration was prepared (VM, from the same lot). The dilution of this stock solution was made depending upon the sensitivity of the instrument. Another stock solution of VM, of concentration equal to the first, was prepared to which a weighed quantity of the calcium salt (perchlorate or thiocyanate) was added to make a solution containing VM and calcium ions in the molar ratio 1:15. When this was mixed, in calculated quantities, with the free VM solution, solutions of different molar ratios were prepared. After thorough mixing over the rotor mixer, the solutions were left for 3–4 h of incubation before use. However, for the ^{13}C NMR titrations, a highly concentrated calcium salt solution in CD_3CN was added directly to the solution containing VM only, and the concentrations of VM in solution were corrected for the dilution when calculating the molar concentration ratios.

The CD and NMR measurements were carried out only in acetonitrile solvent as the calcium salt was found to form a good complex with VM in this solvent. The use of nonpolar solvents like $CDCl_3$ or CCl_4 was not practicable owing to the very poor solubility of the calcium salt in these solvents. Addition of the calcium salt solution in methanol-d_3 or dimethyl-d_6 sulfoxide to the VM solution in these solvents did not show any noticeable change in the spectra of VM.

Results

CD Studies. Marked changes were observed in the CD spectrum of the free VM in acetonitrile on stepwise addition of the solution containing excess of the calcium perchlorate salt in VM. The CD spectra for 13 different molar concentration ratios of VM to calcium salt (R) are shown in Figure 1. The small CD molar ellipticity at 217.5 nm for the free VM, $[\theta]_{217.5} \simeq 4000$ deg·cm²·dmol⁻¹, gradually changes into an intense negative band of molar ellipticity $[\theta]_{217.5} \simeq 12650$ deg·cm²·dmol⁻¹ at $R \simeq 1{:}1.4$. A small CD band, around 240 nm, with positive molar ellipticity is also seen at high concentration of the salt. The change in the molar ellipticity values between VM–calcium and the free VM monitored at 217.5 nm, $[\theta]_{217.5}$, is rather sharp initially up to $R = 1{:}0.5$; thereafter, the changes are small, reaching a saturation value around $R \simeq 1{:}1.5$. It was observed that further addition of the salt solution in large excess, i.e., beyond $R = 1{:}3$, caused the negative molar ellipticity value of the 217.5-nm band to decrease substantially, reaching a minimum (plateau) value at $R = 1{:}15$. The titration graph showing the difference in the molar ellipticities between the free and the (intermediate) complex ($\Delta[\theta]_{217.5}$) vs. the molar concentration ratio (R) is shown in Figure 2. As can be seen, the slope of $\Delta[\theta]$ vs. R is much greater than one would expect for a simple 1:1 stoichiometric complex. The Scatchard plot (Reuben, 1973) (Figure 3) indicates clearly the presence of multiple complexes in solution (see the discussion below).

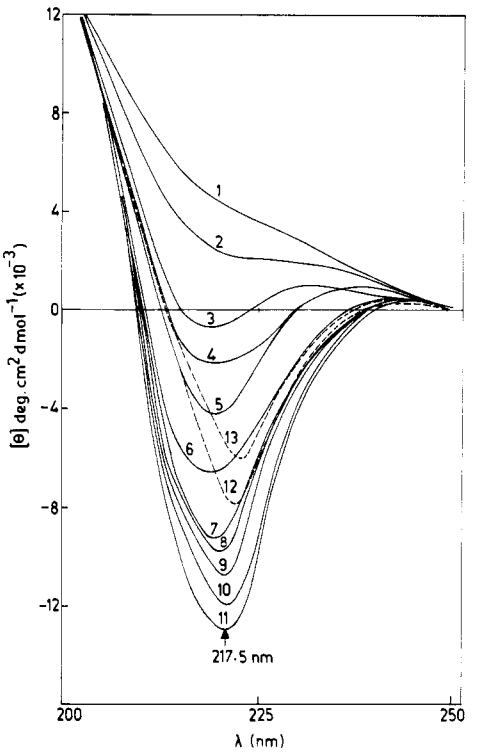

FIGURE 1: CD spectra of VM and VM–calcium in CH_3CN at various molar concentration ratios of VM to calcium (R): $R \simeq$ (1) 1:0.0, (2) 1:0.07, (3) 1:0.15, (4) 1:0.275, (5) 1:0.34, (6) 1:0.46, (7) 1:0.51, (8) 1:0.63, (9) 1:0.70, (10) 1:1.20, (11) 1:1.41, (12) 1:6.00, and (13) 1:15.00. [VM] = 1.22 mM.

Addition of doubly distilled water to the solution containing VM and the calcium salt in the molar ratio 1:1.5 showed a small red shift (with respect to the 217.5-nm band) in addition to a reduced band intensity. In the presence of water, greater than 0.08 mole fraction, the CD spectrum of the solution (R = 1:1.5) had the appearance close to that of the free VM in highly polar solvents (Rose & Henkens, 1974).

The CD spectra were also recorded with the addition of potassium perchlorate solution, also in acetonitrile, to the solution containing VM and the calcium salt in the ratio 1:1.5. Figure 4 shows the effect on the CD spectra of such an addition, for a few concentrations of the potassium salt. The spectra for the solution containing the potassium in a molar

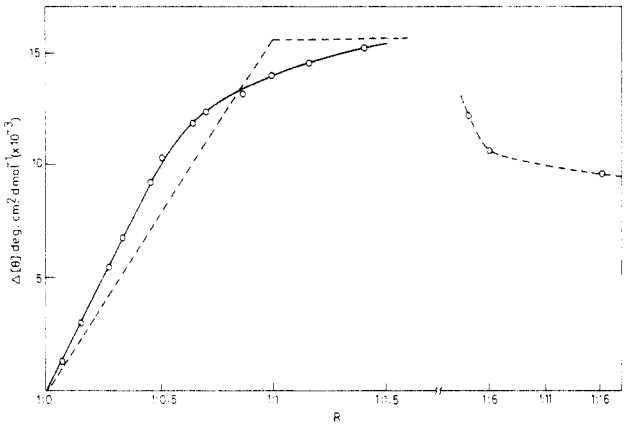

FIGURE 2: CD titration graph of VM–calcium in CH_3CN; [VM] = 1.22 mM.

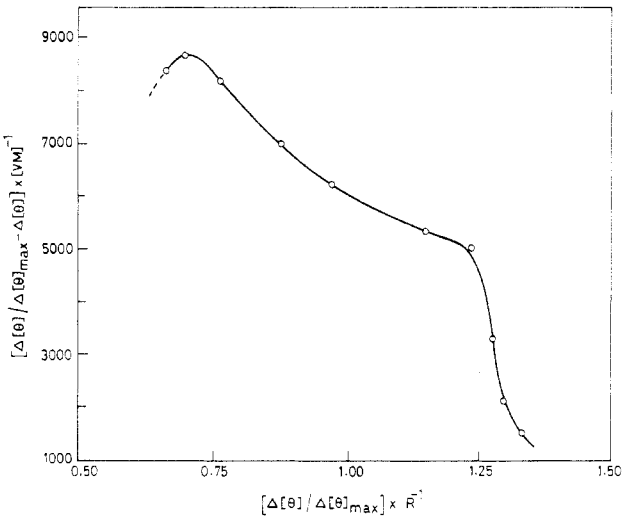

FIGURE 3: Scatchard plot for $0 \leq R \leq 1.5$.

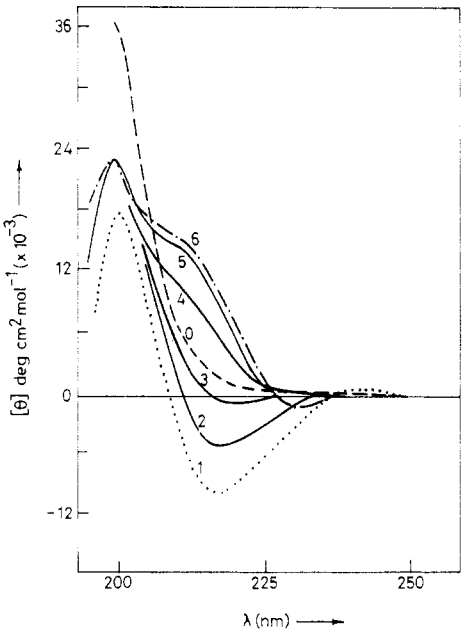

FIGURE 4: CD spectra showing the effect of addition of $KClO_4$ solution to VM–calcium in CH_3CN. [VM] = 0.8 mM; [VM]:[Ca^{2+}]:[K^+] = (0) 1:0:0, (1) 1:1.5:0, (2) 1:1.5:0.3, (3) 1:1.5:0.5, (4) 1:1.5:0.7, (5) 1:1.5:1, and (6) 1:0:1.

ratio, VM:potassium, >1:1, in VM–calcium solution, were found to be identical with the spectrum of VM containing the potassium alone. Due to the excessive absorption of the

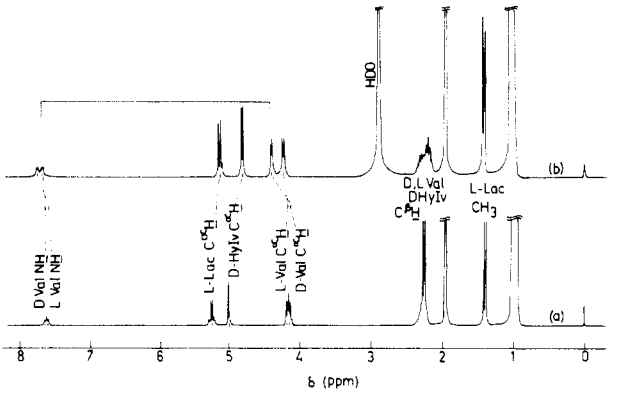

FIGURE 5: 270-MHz ^1H NMR spectra of (a) free VM and (b) VM–calcium perchlorate (R = 1:10.0) in CD_3CN. [VM] = 19 mM; temperature = 25 °C.

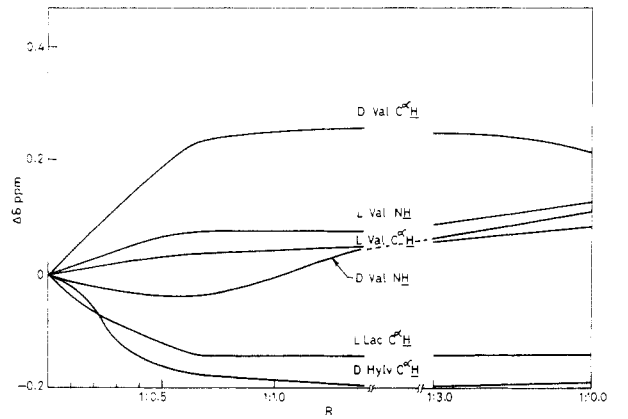

FIGURE 6: ^1H NMR salt titration graph of VM–calcium in CD_3CN; [VM] = 19 mM.

thiocyanate chromophore in the region of 200–250 nm, no CD study could be made with the thiocyanate salt of calcium.

^1H NMR Studies. The 270-MHz ^1H NMR spectra of the free VM and VM–calcium, at R = 1:10.0, in CD_3CN are shown in Figure 5. The assignments of the various proton signals of the free VM in CD_3CN, indicated in the figure, are the same as have been reported (Ovchinnikov & Ivanov, 1974; Bystrov et al., 1977). The assignments shown in the figure for VM–calcium were made through the salt titration and the double-resonance experiments (see below). The ^1H NMR salt titration graphs (Figure 6) obtained through the salt titration experiments at room temperature (25 °C), along the lines detailed in the CD studies, clearly indicate that the salt-induced changes in the chemical shifts are different for different protons. While the L-Val C^α-proton signal remains sharp and unshifted on addition of the calcium salt, the D-Val C^α-proton signal becomes broad and moves continuously downfield rather rapidly till around R = 1:0.5 (Figure 7a). The $\Delta\delta$, the difference in chemical shifts between the chemical shift values of the signals of VM–calcium and the free VM, beyond this molar ratio (R = 1:0.5) is very small and reaches a stabilized value around R = 1:1.5. The C^α-proton signals of the D-Hyi and L-Lac residues broaden initially and shift continuously upfield. The stabilization of $\Delta\delta$ for these signals occurs practically around R = 1:0.5, at which molar ratio the signals also become sharp. The salt addition broadens the D-Val NH signal initially. This signal shifts upfield gradually till around R = 1:0.5. Around this R, the direction of the shift for the signal gets reversed and finally, for large concentrations of the salt, goes to the lower field with respect to its position for the free VM (Figure 7a). On the other hand, the L-Val NH signal continuously shifts downfield with respect to its position for

Table I: ^{1}H NMR Chemical Shifts of VM and VM–Calcium in CD_3CN

	chemical shift (δ) with respect to Me_4Si								
	NH		$C^{\alpha}H$				$C^{\beta}H$	$C^{\beta}H_3$	$C^{\gamma}H_3$
species	D-Val	L-Val	L-Val	D-Hyi	D-Val	L-Val	D-, L-Val and D-Hyi	L-Lac	D-, L-Val and D-Hyi
VM	7.60	7.58	5.24	4.98	4.13	4.16	2.19–2.34	1.39	0.92–1.03
VM–calcium (1:0.5)	7.57	7.65	5.11	4.80	4.36	4.18	2.15–2.34	1.40	0.92–1.05
VM–calcium (1:1.5)	7.56	7.66	5.10	4.79	4.38	4.19	2.11–2.33	1.40	0.92–1.08
VM–calcium (1:10)	7.62	7.68	5.10	4.78	4.36	4.20	2.08–2.33	1.40	0.92–1.08

the free VM on addition of a large excess of the calcium salt to the VM solution ($R > 1{:}6$); further changes in $\Delta\delta$ are noticed, particularly for the C^{α}-proton signals and the NH signals of L-Val and D-Val. The C^{α}-proton signal of D-Val starts moving upfield from the position where it had stabilized at $R = 1{:}1.5$, and that of L-Val, which had practically no shift till $R = 1{:}1.5$, starts moving downfield. The signals of both the NH's shift further downfield. Around $R = 1{:}18$, the NH signals completely overlap, giving a single doublet. At this molar ratio, the C^{α}-proton signals of L- and D-Val overlap to give a broad multiplet. The spectral pattern remained practically the same for further additions of the salt.

The spectra recorded at −30 °C for intermediate concentration ratios (Figure 7b) showed clearly two sets of signals for the D-Val NH and the C^{α} protons of the L-Lac, D-Hyi, and D-Val residues for concentration ratios less than $R = 1{:}0.5$. One set of signals was exactly at the same position as the corresponding set of signals of the free VM in CD_3CN while the other set, presumably due to a VM–calcium complex species, was shifted with respect to the free position. At room temperature (25 °C), the chemical shift position of any particular signal, for a given molar concentration ratio R, was in the average position of the corresponding signals of the two sets at the low temperature. As seen in Figure 7b, the ratio of the intensity of the signal from the free VM species to the intensity of the signal from the complex species continuously decreased with the increase in the calcium salt concentration until $R = 1{:}0.5$. Beyond this ratio, the signals of the set corresponding to the free species practically disappear. It is also noteworthy that the signals of the L-Val C^{α} proton and the NH, at −30 °C, did not show separate signals for the two species and remained sharp throughout. Also, the low-temperature salt titration showed clearly that, in the complex, the lower field C^{α} proton and the upper field NH belonged to the same residue, namely, D-Val.

The ^{1}H NMR salt titration, at room temperature (25 °C), using the thiocyanate salt of calcium instead of the perchlorate salt, gave results nearly identical as in the case with the perchlorate salt except for the fact that the signals of the C^{α} protons of both D-Val and L-Val were a bit broader and the $\Delta\delta$ changes appeared to stabilize around $R \simeq 1{:}1.5$, with no indication of stabilization at a lesser ratio. Similar destabilizing effects, as observed in the case of perchlorate salt, were also observed for the large excess of the thiocyanate salt.

$^3J(HN{-}C^{\alpha}H)$ and $^3J(HC^{\alpha}{-}C^{\beta}H)$ proton spin–spin coupling constants were measured for the free VM and for the intermediate molar concentration ratios (wherever the resolution permitted). The $^3J(HN{-}C^{\alpha}H)$'s for the Val signals were obtained from the corresponding NH signals while the $^3J(HC^{\alpha}{-}C^{\beta}H)$'s of the Val signals were obtained by decoupling the corresponding NH's. Also, the double resonance experiments clearly showed that the outer (lower field) C^{α}-proton signal of a Val residue and the inner NH signal are coupled to the inner C^{α}-proton signal of another Val residue. As the inner NH and valyl C^{α}-proton resonance belong to the D-Val residue (a conclusion arrived at as described above and also through the follow-up in the salt titration experiments), automatically the outer NH and valyl C^{α}-proton resonances could be assigned to the L-Val residue. The ^{1}H NMR parameters

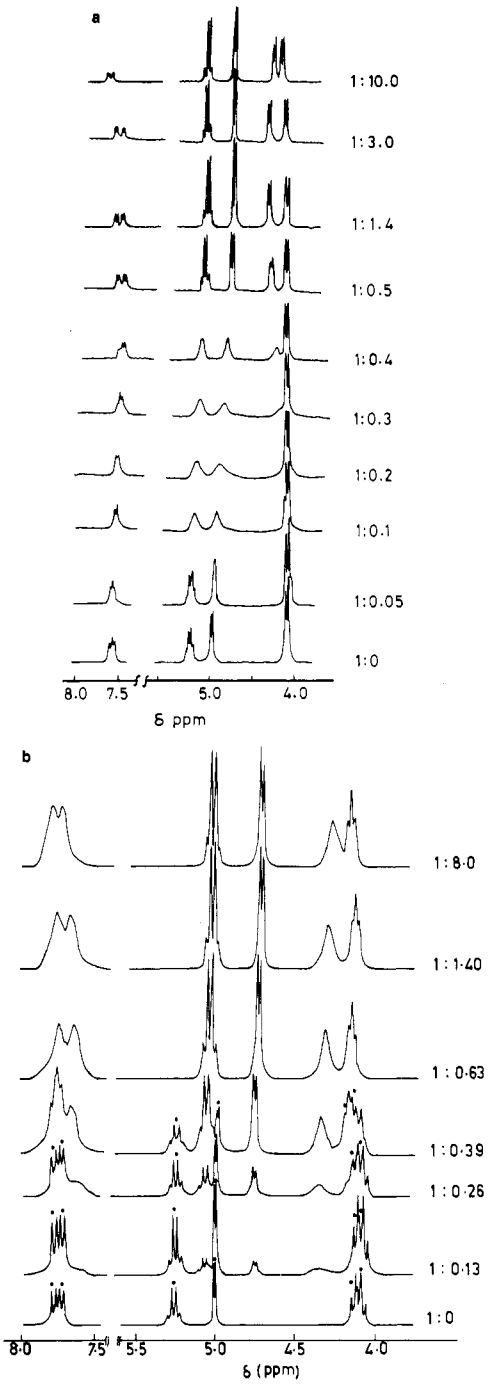

FIGURE 7: ^{1}H NMR spectra ($C^{\alpha}H$ and NH regions) of VM–calcium perchlorate at various molar concentration ratios in CD_3CN at (a) room temperature (25 °C), [VM] = 19 mM, and at (b) low temperature (−35 °C), [VM] = 6.6 mM [the signals of free species are shown as (•)].

Table II: Proton-Proton Spin Coupling Constants of VM and VM-Calcium in CD_3CN

	coupling constant (Hz)					
	$^3J(HN-C^{\alpha}H)$		$^3J(HC^{\alpha}-C^{\beta}H)$			$^3J(HC^{\alpha}-C^{\beta}H_3)$,
species	D-Val	L-Val	D-Val	L-Val	D-Hyi	L-Lac
VM	8.40	7.50	8.70	9.80	3.68	6.99
VM-calcium (1:0.5)	6.99	5.88	5.95	8.05	5.51	7.35
VM-calcium (1:1.5)	6.99	5.52	5.52	7.35	5.88	7.87
VM-calcium (1:10)	5.80	5.50	6.54	7.56	5.85	6.75

for the free VM and for the VM complexes at a few R's are given in Tables I and II.

The temperature coefficients of the chemical shifts, $\Delta\delta/\Delta t$, for the free VM in CD_3CN studied in the temperature range −30 to 50 °C are 3×10^{-3} and 3.5×10^{-3} ppm/°C for the D-Val NH and the L-Val NH, respectively. The $\Delta\delta/\Delta t$, for the same range, for the NH signal of *N*-methylacetamide in CD_3CN (taken as the standard) is 6.4×10^{-3} ppm/°C. The decreased temperature coefficients of the chemical shifts suggest that all the NH's are solvent shielded and/or intramolecularly weakly hydrogen bonded. In the case of VM–calcium ($R \simeq 1{:}1.5$), the temperature coefficients of chemical shifts for both the D- and L-Val NH's are nearly the same, viz., 2.95×10^{-3} ppm/°C. These values indicate that even in the complex, the NH's are intramolecularly hydrogen bonded or solvent shielded. However, at this ratio, as will be shown later, both 1:0.5 and 1:1 complex species coexist, and the temperature coefficients reflect the average value for the two conformers of the existing species. Also, at this concentration ratio even the C^{α}-proton signals of L-Val and D-Val show a temperature dependence, presumably due to the temperature-dependent shift in the chemical equilibria. As such, $\Delta\delta/\Delta t$ values of the NH's do not truely reflect the state of the hydrogen bonds and are masked by the shift in equilibria of the conformers.

D_2O exchange studies on the complex ($R \simeq 1{:}1.5$) could not yield a definitive conclusion regarding the solvent exposition or intramolecular hydrogen bonding of the NH's, as even a small amount of D_2O added to the solution of VM–calcium gave a spectrum very similar to the spectrum of the free VM in polar solvents. Added D_2O, perhaps, destabilized the complex (cf. the CD studies above).

^{13}C NMR Studies. The proton noise decoupled 67.89-MHz ^{13}C NMR spectrum of the free VM in CD_3CN showed significant chemical shift changes, particularly in the carbonyl region of the spectrum, on gradual addition of the calcium perchlorate solution in CD_3CN. The ^{13}C spectral assignments for the free VM in CD_3CN were based on the solvent titration experiment (Figure 8) in which CD_3CN was gradually added to VM solution in $CDCl_3$ or $CDCl_3$ to VM solution in CD_3CN for getting the solvent systems of intermediate compositions. The ^{13}C signal assignments of the free VM in $CDCl_3$ are available in the literature (Bystrov et al., 1977). The changes in ^{13}C chemical shift values, with respect to the values reported for $CDCl_3$, were followed to yield the final assignments of signals with CD_3CN. In spite of the nonideality of the mixed solvent systems, the assignments for the free VM in CD_3CN were straight forward. The assignments of the ^{13}C signals of the carbonyl carbons for free VM in CD_3CN are shown in Figure 9 (cf. Figure 8 and Table III). The assignments of the ^{13}C signals for the complex were done through the salt titration. Some of the typical spectra, for the carbonyl region

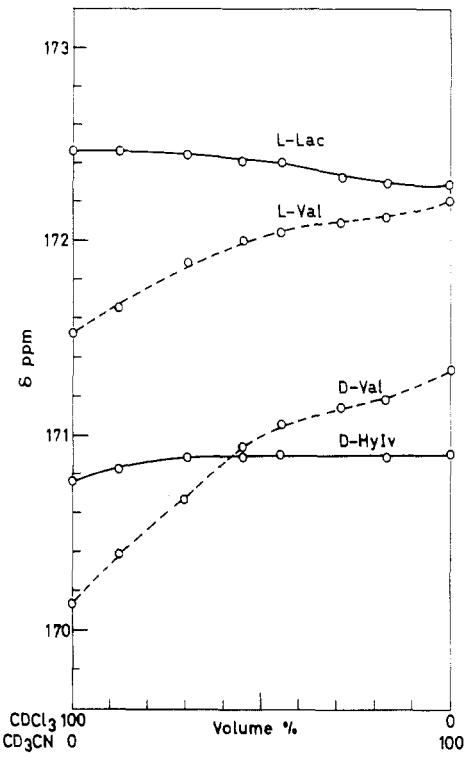

FIGURE 8: Solvent titration graph for the assignment of carbonyl signals of free valinomycin in CD_3CN. *x* axis: volume percent of $CDCl_3$ or CD_3CN. *y* axis: δ with respect to Me_4Si.

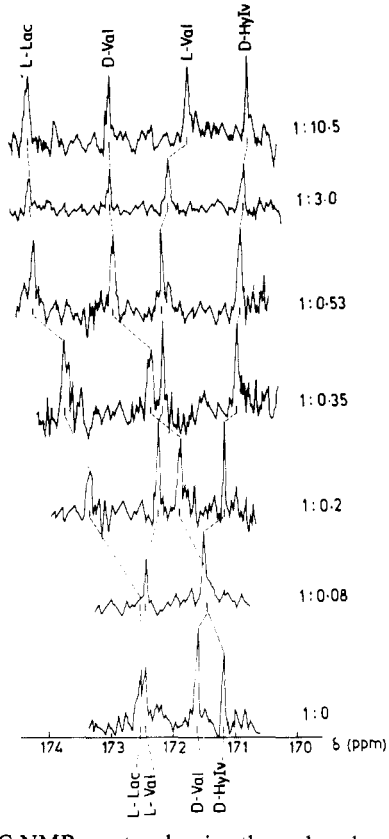

FIGURE 9: ^{13}C NMR spectra showing the carbonyl region for VM–calcium perchlorate in CD_3CN for various R's; [VM] = 32 mM.

only, for various R's are given in Figure 9. On addition of a small amount of the calcium salt ($R < 1{:}0.1$), the signals in the carbonyl region corresponding to L-Lac and D-Val were broadened. With further addition of the salt, the carbonyl carbon signals of L-Lac and D-Val continuously shifted downfield, reaching a saturation value around $R \simeq 1{:}0.5$.

Table III: ^{13}C NMR Chemical Shifts of VM and VM–Calcium in CD_3CN

	chemical shift (δ) with respect to Me_4Si									
	carbonyl				C^{α}–O		C^{α}–N			
species	D-Val	L-Val	L-Lac	D-Hyi	D-Hyi	L-Lac	D-Val	L-Val	$C^{\beta}H$	$C^{\gamma}H_3$
VM	171.64	172.50	172.64	171.20	79.55	71.39	60.30	59.80	31.50; 30.26; 30.26	
VM-calcium (1:0.5)	172.80	172.21	174.32	170.94	79.92	71.58	60.64	59.84	31.68; 30.54; 30.41	17.98–19.37
VM-calcium (1:1)	172.82	172.15	174.31	170.88	79.89	71.59	60.58	59.82	31.62; 30.47; 30.35	17.95–19.31
VM-calcium (1:10)	173.00	171.40	174.40	170.30	79.80	71.39	60.51	59.55	31.62; 30.47; 30.35	17.95–19.31

Small upfield shifts were observed for the carbonyl carbon-13 signals of L-Val and D-Hyi. The changes, $\Delta\delta$, of the ^{13}C signals of the carbonyl carbons of D-Val and L-Lac showed a near-saturation value around $R \simeq 1{:}0.5$. All the signals become sharp at this molar ratio. The changes in values beyond this ratio are minimal for the D-Val and L-Lac carbonyl carbon signals. However, for the large concentration of the salt, further small changes in the ^{13}C chemical shifts were noticed for the L-Val and D-Hyi carbonyl carbons. Table III gives the ^{13}C NMR chemical shift values for the free VM in $CDCl_3$ and for the free VM and a few concentration ratios of the VM to the calcium salt in CD_3CN.

Discussion

CD Studies. The strikingly different nature of the CD spectra observed for VM–calcium as compared to that for the VM–K^+ complex (Figure 4) indicates the different conformations for VM in the two complexes. The rapid increase in the negative molar ellipticity at 217.5 nm up to $R = 1{:}0.5$ and only small changes thereafter suggest that the complex stabilized could be of the stoichiometry 2:1. From the nature of the Scatchard plot (Figure 3) for $R < 1{:}3$, up to which at least no disruptive tendency is noticed, it is obvious that the complex is not a simple 1:1 type. A comparison of the observed Scatchard plot with the theoretically computed Scatchard plots (Reuben, 1973) suggests the coexistence of at least two species of the complexes of the types: VC and V_2C (V = VM; C = Ca^{2+}). The stability constant calculations using the CD titration curve data and assuming simple binding (Rose & Henkens, 1974) or the curve-fitting procedure for the 1:1 complex (Luigi et al., 1980; Vishwanath & Easwaran, 1981) did not give any physically meaningful values.

Assuming the coexistence of VC- and V_2C-type complexes, guided by the nature of the observed Scatchard plot for $R < 1{:}3$, the following relations for the two equilibria could be defined and used for evaluating the binding constants of the species:

$$V + C \underset{k_1}{\rightleftharpoons} VC \qquad k_1 = [V][C]/[VC] \qquad (1)$$

$$VC + V \underset{k_2}{\rightleftharpoons} V_2C \qquad k_2 = [VC][V]/[V_2C] \qquad (2)$$

where [V], [C], [VC], and [V_2C] are respectively the molar concentrations of the uncomplexed free VM, the uncomplexed cation, the species VC, and the species V_2C; k_1 and k_2 are the dissociation constants for the two equilibria. If $\Delta\theta_{VC}$ and $\Delta\theta_{V_2C}$ are the limiting shifts corresponding to the two species VC and V_2C, respectively, then $\Delta[\theta]$ may be calculated with the relation

$$\Delta[\theta] = [VC]\Delta\theta_{VC}/V_t + 2[V_2C]\Delta\theta_{V_2C}/V_t \qquad (3)$$

where V_t is the total VM concentration (held constant) in molar units.

For a given set of k_1 and k_2 and for a known C_t, the total calcium concentration in molar units, the molar concentrations [V], [VC], and [V_2C] can be calculated (Reuben, 1973).

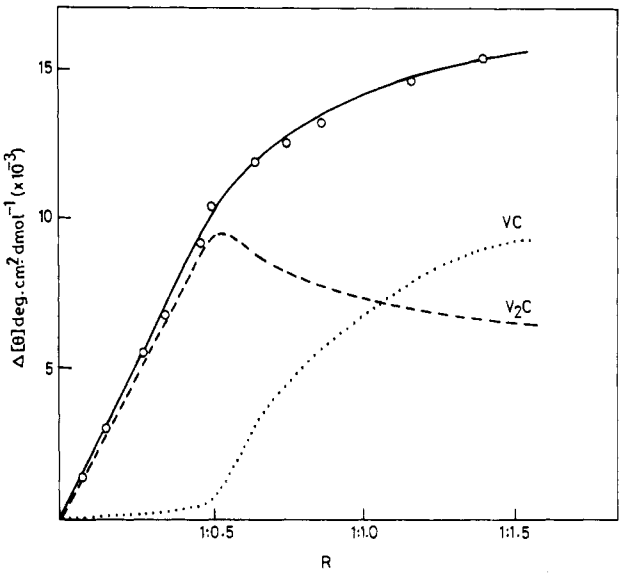

FIGURE 10: Analysis of the CD spectra for $0 \leq R \leq 1.5$: the calculated total spectra (—); the experimental points (O); the contributions from the complex species VC (···) and that from the species V_2C (---) are shown separately.

Hence $\Delta[\theta]_{calcd}$ can be calculated with relation 3 provided $\Delta\theta_{VC}$ and $\Delta\theta_{V_2C}$ are known or assumed. For data where no clear plateau regions are defined, as in our case, the exact limiting values in $\Delta[\theta]$'s for the two species are best obtained by integrating over a range of $\Delta\theta$ values. The values of the dissociation constants for the two species, k_1 and k_2 in molar units, are also best obtained from a nonlinear curve fit made by the proper choice of a set of values for k_1, k_2, $\Delta\theta_{VC}$, and $\Delta\theta_{V_2C}$, the best fit achieved by varying these four parameters until the root mean square difference, $\Delta\theta_{RMS}$, defined below is minimum:

$$\Delta\theta_{RMS} = \sum_{i=1}^{N}(\Delta[\theta]_{calcd_i} - \Delta[\theta]_{obsd_i})^2/N$$

The minimization was effected, with a computer program written by the authors, and a best fit was obtained for the set: $k_1 = 2.0 \times 10^{-3}$ M, $k_2 = 1.0 \times 10^{-3}$ M, $\Delta\theta_{VC} = -2.48 \times 10^4$ deg·cm²·dmol⁻¹, and $\Delta\theta_{V_2C} = -1.0 \times 10^4$ deg·cm²·dmol⁻¹ with root mean square difference $\Delta\theta_{RMS} = 1.64 \times 10^2$ deg·cm²·dmol⁻¹. The corresponding binding constants for the species VC and V_2C are 500 M^{-1} and 1000 M^{-1}, respectively. Figure 10 shows the theoretically computed CD titration graph along with the experimentally observed data points. This CD titration graph analysis makes apparent the stoichiometry of the complexes, viz., (a) predominantly a 2:1 complex for the concentration ratios less than 1:0.5 and (b) the coexistence of 1:1 and 2:1 complexes for the concentration range $1{:}0.5 \leq R \leq 1{:}1.5$.

The reduction in the $\Delta[\theta]$ for R's greater than 1:3 and a CD spectrum, at large salt concentration, similar to the one observed for VM in polar solvents (Rose & Henkens, 1973) may be due to the breaking of the structure or the assumption

of a rather labile open conformation for the complex.

It is interesting to compare the binding constant of the VM–K^+ complex in CH_3CN, 3×10^5 M^{-1} (Rose & Henkens, 1973), with the binding constants of the VC and the V_2C types of complexes, 5×10^2 M^{-1} and 10^3 M^{-1}, respectively. This is not suprising in view of the well-known selectivity of VM to K^+ over other metal ions, divalent or monovalent. This preference of VM to K^+ over Ca^{2+} is substantiated by the titration experiment (Figure 4) where $KClO_4$ was added to the solution of the VM–calcium complex ($R \simeq$ 1:1.4). The spectral parameters, viz., $\Delta[\theta]$ and the wavelength at which the CD band corresponding to $n \rightarrow \pi^*$ occurs, gradually changed to a limiting value, which was identical with that expected for VM–K^+ in CH_3CN. The selectivity for K^+ over Ca^{2+} could be accountable predominantly in terms of the differences in (a) the free energy of the solvation of Ca^{2+} and K^+, (b) the conformation of the depsipeptide part of the two complexes, and (c) the charged states of the metal ions and the liganding dipolar carbonyl groups. The double-charged Ca^{2+} could be expected to polarize more strongly the liganding groups than the single-charged K^+. This, if considered alone, should make the binding constant of VM–calcium more than that for VM–K^+. However, this contribution is more than compensated for by the contributions from the differences in solvation energy and in the conformational states of VM in the two complexes. For example, the solvation energy of Ca^{2+} in CH_3CN (=−350.0 kcal/g of ion) is almot 5 times that of K^+ (=−75 kcal/g of ion) (Burgess, 1978) and hence makes a substantial contribution to the decreased stability of VM–calcium complexes, the displacing of the solvent sheath around the metal ion by the liganding groups being far easier in the K^+ case than in the Ca^{2+} case. Apparently, the depsipeptide molecule with all its carbonyl pointing inward toward the center in VM–K^+ competes better in terms of the complexation with the cation over the solvation of the ion than in the case of VM–calcium where the liganding carbonyls hold the Ca^{2+} in a disposition exposing the ion for sufficient interaction with the solvent.

NMR Studies. A comparison of the ^1H NMR data for the free VM in different solvent systems (Ovchinnikov & Ivanov, 1974; Patel & Tonelli, 1973; Bystrov et al., 1977) indicates that both the chemical shifts and the coupling constants of the free VM in CD_3CN are closer to the ones reported for the nonpolar solvents like $CDCl_3$ than to those of the medium polar solvents (e.g., a CCl_4 plus dimethyl-d_6 sulfoxide mixture, 3:1 v/v) or the more polar solvents (e.g., CD_3OH and dimethyl-d_6 sulfoxide). Thus, the conformation of the free VM in CD_3CN could be assumed to be closer to the "A_2-bracelet" conformation (Ovchinnikov et al., 1974). The temperature coefficients of the chemical shifts for the NH's of the valyl residues of VM in CD_3CN also give an indication of the existence of the six weak intramolecular hydrogen bonds of the $4 \rightarrow 1$ type present in the A_2-bracelet conformation. This is further supported by the studies using the 2,2,6,6-tetramethylpiperidinyl-1-oxy free radical, where the addition of the free radical to the solution of VM in CD_3CN did not show noticeable broadening of the NH signals (S. Devarajan, private communication). In what follows, we take the conformation of the free VM in CD_3CN as the A_2-bracelet type with all the six carbonyls pointing toward the symmetry axis.

From the low-temperature salt titration spectra it is clear that the exchange rate between the free and the complexed VM species is rather slow on the NMR time scale. The comparison of spectra at −30 °C with those at room temperature (25 °C) shows that the broadened signals at room temperature of the L-Lac, D-Val, and D-Hyi C^α protons and the D-Val amide proton are the exchange-broadened average signals. In fact, at higher temperatures (50 °C), only one set of sharp signals appears even at low concentrations of the salt. This shows that the rate of exchange between the free and the complexed species of VM could be enhanced by elevating the temperature. Even when the tendency of the formation of different stoichiometric complexes and the possibility of their coexistence exist, as seen above, the signals remain sharp after R = 1:0.5. This indicates clearly that the exchange between the different stoichiometric complexes, beyond R = 1:0.5, must be fast on the NMR time scale. It must, however, be noted that, at all temperatures and for all the concentration ratios studied, the NMR spectra of VM–calcium show that the C_3 symmetry is retained in this system also.

Determination of solution conformation of the VM complex(es) with calcium could be attempted by comparing the solution data of VM–calcium with those of VM–K^+ for which the solution conformation has been firmly established (Shemykin et al., 1969; Bystrov et al., 1977; Ohinishi & Urry, 1969). The limiting values of the spectral parameters of both the CD and the NMR are arrived at for VM–K^+ sharply around R = 1:1, indicating that VM forms an equimolar complex with K^+ (Rose & Henkens, 1974; Ivanov et al., 1975), while only the tendency of ill-defined stabilization regions could be seen over the ranges around $R \simeq$ 1:0.5 and 1:1.5 and $R >$ 1:15 for VM–calcium. Only VM–calcium shows nonmonotonical variations in the chemical shift values of the C^α proton and the amide proton of D-Val (as do the molar ellipticity values in the CD) over the concentration ranges studied. In the VM–K^+ complex, the salt-induced chemical shift changes on the D- and L-Val residues are nearly equal and isodirectional with respect to the C^α protons in the ^1H NMR and the carbonyl carbons in the ^{13}C NMR (Bystrov et al., 1977), indicating that the complexed cation is symmetrically placed with respect to the D- and L-Val residues. In contrast to this, only the C^α proton of D-Val shifts, to the lower field, appreciably in the complex(es) of VM with calcium while the carbonyl carbon-13 signals of D- and L-Val shift in the opposite direction and to different extents. Again, in the case of the VM–calcium, the upfield shifts are noticed for the D-Hyi and L-Val carbonyl carbons; the L-Lac carbonyl carbon shifts downfield nearly as much as the D-Val carbonyl carbon does. Further, the changes in the chemical shifts and in the coupling constant values $^3J(HN-C^\alpha H)$ are all smaller for VM–calcium compared to those for the VM–K^+ complex.

The above features of the NMR data would immediately suggest that the complexation of VM with calcium is of quite a different nature compared to that of VM–K^+, i.e., in terms of the coordination, the stoichiometry, and the conformation. The evidence for the stoichiometry of 2:1 for the VM–calcium complex comes from the comparatively stronger tendency of stabilization of the $\Delta\delta$ values around R = 1:0.5 and near complete disappearance of the free signals around this ratio in the low-temperature salt titration experiment. However, the possibility of the existence of a 1:1 equimolar complex is also suggested by the gradual changes, albeit small, even beyond R = 1:0.5 until around 1:1.4. Also, the slope $\Delta(\Delta\delta/\Delta R)$ for $1{:}0 \leq R \leq 1{:}1.5$ shows an intermediate value between those expected for 2:1 and 1:1 complexes. The changes that are observed for very large salt concentrations could be suggestive of breaking of the earlier stabilized complexes due to the excess salt present or to the coexistence of higher stoichiometric complexes or to a labile open conformation of a complex species.

The explanation for the observed changes in the NMR spectral parameters when calcium is added to VM in CD_3CN is to be sought in terms of the conformational changes from the A_2 bracelet of the free VM, the ion–dipole interactions between the calcium ion and the liganding groups participating in the ion binding, and the various consequential secondary effects. As the direct action of the metal with the coordinating carbonyl oxygens is expected to shift the carbonyl carbon-13 chemical shifts to downfield due to stronger polarization along the C=O bond resulting in the reduction of the electron density around the carbon, the coordination of the ion with the L-Val and D-Hyi carbonyl groups would be easily ruled out. The shifts to the low field of the carbonyl carbon-13 signals of D-Val and L-Lac suggest that the binding of Ca^{2+} may be on the side of the VM bracelet where they are located and that, also, possibly they are involved in the ion binding. The observed changes in the carbonyl carbon-13 shifts are smaller than those expected for a double charge on the ion [cf. the corresponding shifts for the VM–K^+ complex (Bystrov et al., 1977)], suggesting a weaker coordination from the liganding carbonyls. This weaker coordination may be due to the large deviation in the angle C=O···Ca^{2+} from 180°, resulting in only partial polarization along the C=O bond. The large deviation from 180° of this angle may be explained from considerations of the electrostatic repulsion between the coordinating ligands as they come closer to hold the calcium than when binding the larger sized ions like K^+. The positively charged Ca^{2+} possibly sits above the plane formed by the three oxygens of the oriented carbonyls. The actual orientation of the liganding carbonyls could be expected to be determined by the combined effects of the electrostatic repulsion among the carbonyl oxygens and the electrostatic attraction between the cation and the carbonyl oxygens.

The observation from the titration experiments, namely, VM–calcium shows a stronger tendency to stabilize at the stoichiometric ratio 2:1 (i.e., R = 1:0.5), allows one to conjecture that the cation is sandwiched between the two VM molecules. The complexing side of VM appears to be the side of the bracelet on which the D-Val and L-Lac residues are located. The preference to this side over the "L-Val-D-Hyi side" of the bracelet may be due to the steric hindrance, from the bulkier side groups of the L-Val and D-Hyi residues, encountered by cation when entering from this side. The comparatively large downfield shifts of the carbonyl carbon-13 signals of D-Val and L-Lac support the idea of the complexation on the "D-Val-L-Lac side". The "sandwich model" of a 2:1 complex with the D-Val carbonyls, three each from the two VM molecules providing the main coordination for the smaller sized cation Ca^{2+}, could be seen to be acceptable from the model-building studies. The orientation of the liganding carbonyls of D-Val would be outward. The change in the orientation of the carbonyls of D-Val would position the C^α proton of D-Val in the deshielding cone of the carbonyls, which would explain the downfield shift of the D-Val C^α-proton signal. With regard to the downfield shift of the L-Lac carbonyl carbons, some detailed explanations are needed: the apparent larger downfield ($\simeq$1.7 ppm) shift of L-Lac carbonyls, even greater than that of D-Val's ($\simeq$1.2 ppm), appears at first sight to indicate a stronger coordination from L-Lac carbonyl oxygens to metal ion. However, in the 2:1 complex, the metal ion cannot be expected to be in the interior of either of the symmetrically placed (symmetry with respect to calcium is suggested in the spectrum) VM molecules because of steric hindrances from the side groups of D-Val and L-Lac. It is to be noted that carbonyl oxygens of D-Val and L-Lac of any one VM molecule lie in different planes. With calcium placed outside of the "D-Val-L-Lac sides" of both molecules of VM and on the symmetry axis, it follows from the model-building studies that the carbonyl oxygens of D-Val rather than of L-Lac are nearer to the metal ion. Also the carbonyl C=O's of D-Val, rather than of L-Lac, make an angle closer to 180° with the metal ion. These facts would indicate that L-Lac must have a weaker coordination with calcium. The explanation for its downfield shift, larger than that of D-Val, may be then expected in terms of strengthening or formation of hydrogen bond involving L-Lac carbonyls. The upfield shift of the NH signal of D-Val, however, shows weakening of the (L-Lac) C=O···H–N (D-Val) intramolecular hydrogen bond. The comparatively open L-Lac C=O (because of weakening of intramolecular hydrogen bonding and of weaker coordination with metal ion also) may be then expected to be rather exposed to solvent or water (that has not been completely stripped off the cation, due to the latter's larger solvation sheath). The intermolecular hydrogen bonding of C=O of L-Lac with solvent donor atoms may cause a sufficient downfield shift of L-Lac carbonyl signals. Downfield shifts of the order of 2.8 ppm for the L-Lac carbonyl due to the formation of an intermolecular hydrogen bond with the solvent [with consequent weakening of the (L-Lac) C=O···H–N (D-Val) intramolecular hydrogen bond] have been reported (Bystrov et al., 1977). From the molecular model-building studies, it could be seen that the complexation on the "D-Val-L-Lac side" of sandwiching VM molecules, with the L-Lac carbonyls also providing a weak coordination to the cation, would slightly open the bracelet structure of the uncomplexed "L-Val-D-Hyi side" of the molecules of VM. This can affect adversely the intramolecular hydrogen bonds of the type (D-Hyi) C=O···H–N (L-Val). The upfield shift of the D-Hyi carbonyl carbon-13 signal may possibly be an indication of such weakening of the intramolecular hydrogen bonds. However, the amide proton signal of L-Val shows a downfield shift, suggesting a possible indication of such weakening of the intramolecular hydrogen bonds. However, the amide proton signal of L-Val shows a downfield shift, suggesting a possible strengthening of the hydrogen bond. In such apparently contradicting observations on the state of the hydrogen bond based on the ^1H NMR and ^{13}C NMR, the reliance on the carbonyl carbon shifts appears to be more reasonable for the state of the intramolecular hydrogen bond as the amide proton signal may show a downfield shift even when the proton is solvated with the solvent or the water molecules (which might have entered in minute quantities along with the hygroscopic calcium salts or the solvents added). Then, what is observed as the changes in the amide-signal chemical shifts could be the combined effect of two types of hydrogen bonds involving a single donor (NH) and two acceptors, viz., oxygens of D-Hyi C=O and N of CD_3CN or O of H_2O.

A comparison of the $^3J(HC^\alpha–C^\beta H)$ proton spin–spin coupling constants of VM–calcium (Table II) with those of the free VM and of the VM–K^+ (Bystrov et al., 1977) complex shows that the conformational states of the side chains of the residues in VM–calcium around the $C^\alpha–C^\beta$ bonds are appreciably different from those of the free VM or of VM–K^+. From the simple application of the Karplus-like curve (Kopple et al., 1973) all the $\chi_1(HC^\alpha–C^\beta H)$ angles are found to be 120° for the VM–calcium complex (2:1). Thus, unlike the case of VM–K^+ where the C^β protons of the D-Val and L-Val residues are "trans" to their respective C^α protons and the C^β proton of the D-Hyi residue is "gauche" to its C^α proton, in the case of VM–calcium we have near gauche orientations of the C^β protons for the D-Val, L-Val, and D-Hyi residues with respect

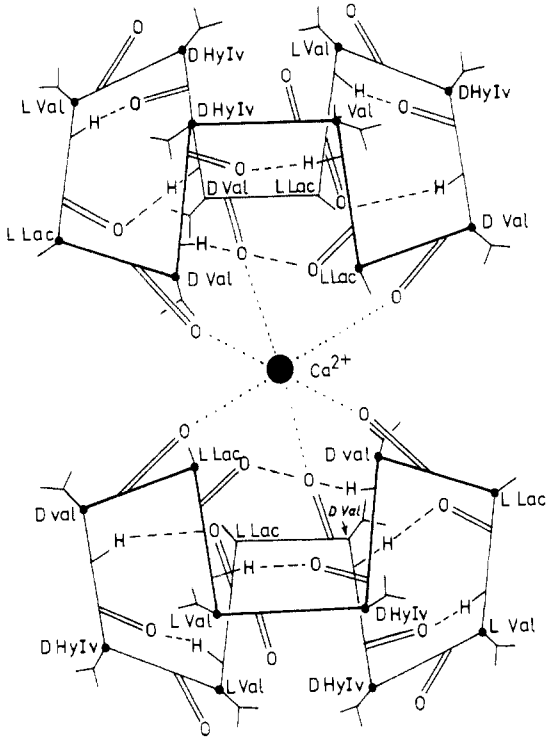

FIGURE 11: Schematic diagram of the proposed model for the sandwich (2:1) complex for valinomycin–calcium complex. [Only strong coordination bonds are shown as (···)].

to their C^{α} protons. These changes in the conformational states of the side chains are probably necessitated by the steric forces that are called into play by the different type of coordination and the slightly opened-out conformation VM–calcium has in its 2:1 sandwich complex as compared to that of the equimolar (1:1) complex of VM–K^+. As the analysis of the CD data indicated, only negligible quantities of other than 2:1 complex are present for $R \leq 1{:}0.5$. The observed vicinal $^3J(HN{-}C^{\alpha}H)$ coupling constants of D-Val and L-Val give an estimation of $\phi(HN{-}C^{\alpha}H)$ for the 2:1 complex (Bystrov et al., 1977). With the estimated values of ϕ for the L-Val and D-Val residues and $\chi_1(HC^{\alpha}{-}C^{\beta}H)$ for the L-Val, D-Val, and D-Hyi residues and with the assumption of little changes for ψ, the model-building studies consistent with the NMR data in respect to intramolecular hydrogen bonds allowed us to arrive at a model (Figure 11) for the 2:1 complex of VM–calcium. The set of dihedral angles (in degrees) used for the model building is

	D-Val	L-Lac	L-Val	D-Hyi
ϕ	60	−60	−60	100
ψ	−100	−90	100	60
χ_1	120		120	120

[IUPAC–IUB Commission on Biochemical Nomenclature (1970)].

The changes, though small, observed in the NMR parameters beyond $R = 1{:}0.5$ and showing a tendency of stabilization around $R \simeq 1{:}1.5$ may be taken to be indicative of VM forming an equimolar complex with the Ca^{2+}. But, unlike the equimolar complex of VM with K^+ where the cation is held at the center by six centrally directed carbonyls, three each from D-Val and L-Val, the equimolar complex of VM and Ca^{2+} has the cation held wholly by the D-Val and L-Lac carbonyl groups, each group providing nearly equal, but small, contributions to the ion binding in terms of the ion–dipole interactions. A plausible explanation for such a gradual switch over from the 2:1 sandwich complex to the "D-Val-L-Lac side

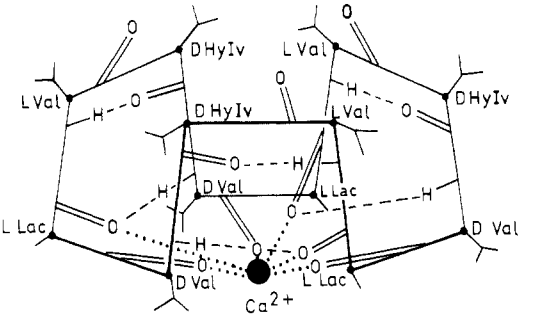

FIGURE 12: Schematic diagram of the proposed model for the "D-Val-L-Lac side" equimolar (1:1) complex for the valinomycin–calcium complex.

equimolar complex" could be that at the initial stages where the concentration of VM is in large excess as compared to the calcium ion, V_2C is formed in preference to VC. As the calcium concentration is increased, the equilibria among the different species should shift in such a way that the utilization of the added calcium would be maximum. Thus in the large concentration range, viz., $0.5 \leq R \leq 1.5$, VC is formed in preference to V_2C. However, the barrier for the conformational change between the two complexes should be very small as indicated by the small changes observed in the NMR parameters. The exchange between the two complexes must be too rapid, on the NMR time scale, to be detected. The only change in the two complexes could possibly be due to a small inward orientation of the D-Val carbonyls. There is no appreciable change in the $^3J(HC^{\alpha}{-}C^{\beta}H)$ values when the R is increased to 1:1.5 from 1:0.5. This allows us to assume very little changes in the conformations of the side chains around the $C^{\alpha}{-}C^{\beta}$ bonds of D-Val, L-Val, and D-Hyi in going from the "sandwich" (2:1) to the "equimolar (1:1) D-Val-L-Lac side complex". Practically, $^3J(HN{-}C^{\alpha}H)$ values of D-Val and L-Val remain the same in the entire range $0.5 \leq R \leq 1.5$, despite the coexistence of the 2:1 and the 1:1 complexes in this range. This suggests that, on an average, the ϕ angles of D-Val and L-Val are nearly the same for both the 2:1 and the 1:1 complexes while only the ϕ angle of L-Lac changes. Thus, one is led to a plausible model for the "1:1 equimolar D-Val-L-Lac side complex" of VM–calcium (Figure 12). The set of dihedral angles (in degrees) for the D-Val-L-Lac side equimolar complexes is

	D-Val	L-Lac	L-Val	D-Hyi
ϕ	60	0	−60	100
ψ	−120	−90	100	60
χ_1	120		120	120

[IUPAC–IUB Commission on Biochemical Nomenclature (1970)].

The changes in the chemical shifts and the coupling constants observed at very large concentrations of the salt ($R >$ 1:15), viz., a downfield shift of the C^{α}-proton signal of L-Val, an upfield shift of the D-Val C^{α}-proton signal with respect to the position at $R \simeq 1{:}1.5$, considerable downfield shifts of both amide proton signals, and further upfield shifts of the L-Val and the D-Hyi carbonyl carbon-13 signals, are difficult to explain. However, the observation that the chemical shift values of all the signals are closer to those of the free VM in polar solvents suggests a labile conformation—an average of many possible indistinguishable conformations on NMR time scale—for the system at large concentrations of the salt. The amide protons show a clear indication of the solvent exposition by the strengthened intermolecular hydrogen bonds while the corresponding carbonyls show clear indication of the broken

intramolecular hydroben bonds. However, the coupling constant values ^3J(HN–C$^\alpha$H) (Table II), which are far less than the expected values for the polar solvents, suggest that the system could still be of a complex that has a rather flat structure—a labile conformation—with no intramolecular hydrogen bonds.

It is noteworthy that on the basis of the CD studies the existence of multiple complexes of VM with another divalent cation, viz., Ba^{2+} (Devarajan & Easwaran, 1981), and that a crystal structure of VM–Ba^{2+} (Devarajan et al., 1980) in a novel 1:2 conformation have been reported from our laboratory.

Conclusions

The detailed analyses of the CD and NMR data of VM–calcium indicate that the solution conformation of VM–calcium complex(es) is quite different from the well-known bracelet type of VM-K^+. The favored conformation for the complex depends on the salt concentration; at the low salt concentration ($R \leq 1{:}0.5$), the proposed conformation is a novel type, viz., 2:1 (peptide–ion–peptide) sandwich. In the salt concentration range $1{:}0.5 \leq R \leq 1{:}1.5$ a mixture of two conformers, namely, a 2:1 sandwich and a "D-Val-L-Lac side" equimolar complex (1:1), seems to coexist. At very high salt concentration ($R > 1{:}15$), the conformation of the complex is more close to a labile open conformation similar to that observed for the free VM in the polar solvents. The findings that VM can also form sandwich complexes, like the depsipeptide antibiotic Enniatins and some analogues of VM (Ovchinnikov, 1974), are interesting in the sense that this fact throws light on the conformational adaptabilities of the free VM when complexing with the cations. When taken in the broader sense, the results may be suggestive of one of the several pathways by which the cations get transported through the membranes; viz., the depsipeptide VM could be forming the intermediate, though less stable, sandwich complex, the equimolar complexes with the cations being held on the "D-Val-L-Lac side" or at the center or on the "L-Val-D-Hyi side" (an extrapolation), and then be opening out into a form at which it can easily release the cation, thus completing a cycle in the cation transport across the membrane. Further the studies indicate that VM can complex with the divalent cation Ca^{2+} even at low absolute concentration of VM, given a proper lipophilic solvent medium and perhaps a stabilizing symmetric anion. Such a complexation and the type of exchange with K^+ as discussed in the text are important in understanding the carrier mechanism by which VM facilitates Ca^{2+} transport, through "Ca^{2+}–$2K^+$ exchange" in biological membranes such as the sarcoplasmic reticulum etc. (Chiu & Haynes, 1980).

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