# Nuclear Magnetic Resonance Studies of the Interactions of Anions and Solvent with Cation Complexes of Valinomycin†

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ABSTRACT: The solution conformation for cation complexes of valinomycin (VAL) were investigated by $^1H$ and $^{13}C$ nuclear magnetic resonance (NMR). NMR techniques were also used to estimate the association constants of VAL for $Na^+$ and $K^+$ in acetone, as well as to measure the rate constants and activation energies for the dissociation of these complexes in both acetone and chloroform. From the results it is concluded that the Na complex but not the K complex may exist in at least three different conformational states depending upon the anion and the polarity of the solvent. As judged by the chemical shifts and vicinal coupling data, the NaBr, $SCN^-$, and trinitro-*m*-cresolate (TNC) complexes of VAL in chloroform have a conformation that is intermediate between that of uncomplexed VAL and KVAL complexes. The conformation of the Na–tetraphenylboron complex, however, is almost identical to that of KVAL. Proton spectra of other cation complexes in chloroform suggest that the structure of the Li complex is like that of Na, whereas complexes of Rb, Cs, $NH_4$, and Tl resemble the K–VAL molecule in structure. In acetone the Na complex has a structure distinct from but close to the conformation of free VAL. On the other hand, the K complex has the same braclet-like conformation it has in chloroform. Structural differences between the Na and K complexes in acetone are reflected accordingly in the cation selectivity of VAL which has association constants of 25 and $>10^4\ M^{-1}$, respectively, for Na and K.

The study of the molecular basis for the ionic permeability of biological membranes has been stimulated by the discovery of small macrocyclic antibiotics such as valinomycin (Ovchinnikov et al., 1972, and references therein). This compound, a cyclic dodecadepsipeptide of microbial origin has the formula: *cyclo*[D-Val-L-Lac-L-Val-D-Hydval]$_3$ and is known to increase selectively the monovalent cation permeabilities of biological (Pressman, 1968; Tosteson et al., 1967) and lipid bilayer membranes (Mueller and Rudin, 1967; Lev and Buzhinskii, 1967; Andreoli et al., 1967). For the most part the ion-selective actions of valinomycin on these membranes parallel the relative magnitudes of the formation constants measured in bulk phases where K, Rb, and Cs complexes are found to be much more stable than Na or Li complexes (Pressman, 1968; Shemyakin et al., 1969; Grell et al., 1972). Recently, however, experiments by Tosteson and coworkers (Davis and Tosteson, 1971; Tosteson, 1972) showed that the cation specificity of the valinomycin-induced conductances and ion permeabilities in lipid bilayer and red blood cell membranes was nearly abolished when the organic anion, trinitro-*m*-cresolate (TNC)[1] was added to the membrane bathing media. Likewise measurements of the partition coefficients for Na and K ions between aqueous salt solutions and organic phases containing valinomycin showed reduced cation selectivities if $TNC^-$ was present in the aqueous phase. It was also found that the optical absorption spectrum of the $TNC^-$ depended upon the solvent polarity and, more significantly, whether Na or K was complexed with valinomycin. These experiments together with some recent findings by Rose and Henkins (1974) showed that for an appropriate choice of anion or solvent, the cation selectivity range of valinomycin could be reduced by increasing the stability of the Na complex possibly via ion-pair formation.

Although there is a considerable body of information about the structural and functional properties of free valinomycin and its $K^+$ ion complex (Haynes et al., 1969; Ivanov et al., 1969; Ohnishi and Urry, 1970; Ivanov et al., 1971; Duax et al., 1972; Krigbaum et al., 1972; Patel and Tonelli, 1973), less is known about the structures of complexes formed from the other alkali metal ions, especially $Na^+$. This gap in our knowledge about the structure of the Na–valinomycin complex is partly a consequence of the fact that until recently (Davis and Tosteson, 1971; Ivanov et al., 1971; Rose and Henkins, 1974) there was little evidence to suggest that Na formed a stable complex with valinomycin, and partly because previous studies were carried out in polar solvents in which the association constant of Na ions for valinomycin is small (Haynes et al., 1971; Grell et al., 1972). Since the structural or conformational properties of valinomycin play a significant role in its potassium-selective binding properties (Pinkerton et al., 1969; Ivanov et al., 1971; Mayers and Urry, 1972; Patel and Tonelli, 1973), structural information about complexes of Na and other monovalent cations is of value and interest. Clearly such information affords broader insight into the conformational basis for cation selectivity in these compounds, as well as some illumination of the problem of ion selectivity in biological membranes. Thus nuclear magnetic resonance (NMR) techniques were used to investigate the structures and, in some cases, the functional properties of valinomycin–cation complexes. Of primary interest was to examine the correlations between structure and cation selectivity and to learn how these correlations are affected by anions and solvent polarity. Because of the importance of Na and K

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[1] Abbreviations used are: TNC, 2,4,6-trinitro-*m*-cresolate; VAL, valinomycin; TPB, tetraphenylborate:

Table I: Proton Chemical Shifts of Valinomycin and Its Cation Complexes in $CDCl_3$.[a,b]

| Salt | NH | | $C_\alpha H$ | | | |
|---|---|---|---|---|---|---|
| | D-Valine | L-Valine | D-Valine | L-Valine | L-Lactate | D-Hyiv[c] |
| | 7.85[d] | 7.75[d] | 4.13 | 3.98 | 5.32 | 5.02 |
| LiTNC | 7.95 | 7.91 | 3.90 | 3.87 | 5.38 | 5.04 |
| NaBr[g] | 8.25 | 8.21 | | | 5.34 | 5.04 |
| NaSCN | 8.22[e] | 8.14[e] | 3.88 | 3.87 | 5.28 | 5.00 |
| NaTNC | 8.26[e] | 8.18[e] | 3.88 | 3.85 | 5.31 | 5.03 |
| NaTPB | 8.36 | 8.30 | 3.94 | 3.97 | 4.97 | 4.61 |
| KCl | 8.30 | 8.27 | 3.86 | 3.83 | 4.93 | 4.58 |
| KSCN | 8.37 | 8.26 | 3.89 | 3.78 | 4.96 | 4.60 |
| KTNC | 8.40[f] | 8.28[f] | 3.86 | 3.82 | 4.89 | 4.57 |
| KTPB | 8.40 | 8.28 | 3.86 | 3.81 | 4.91 | 4.57 |
| RbBr | 8.21 | 8.09 | 3.84 | 3.79 | 4.96 | 4.61 |
| CsCl | 7.98 | 7.88 | 3.82 | 3.76 | 5.05 | 4.69 |
| $NH_4TNC$ | 8.23 | 8.10 | 3.83 | 3.78 | 5.00 | 4.65 |
| TlTNC | 8.18 | 8.08 | 3.88 | 3.88 | 5.13 | 4.76 |

[a] In ppm (±0.02) from $Me_4Si$, spectra were recorded at both 100 and 90 MHz. [b] Concentration of valinomycin: ca. $5-10 \times 10^{-3}$ *M*. [c] D-hyiv, D-hydroxyisovalerate. [d] $5.5 \times 10^{-3}$ (D-Val); $6.8 \times 10^{-3}$ (L-Val); temperature coefficients (ppm/deg). [e] $2.7 \times 10^{-3}$ (SCN); $3.5 \times 10^{-3}$ (TNC); temperature coefficients (ppm/deg). [f] $2.5 \times 10^{-3}$ (TNC); temperature coefficients (ppm/deg). [g] Only 60% of the valinomycin formed a complex with NaBr.

Table II: Proton Vicinal Coupling Constants for Valinomycin and Its Cation Complexes in $CDCl_3$.[a]

| Salt | $J_{NHC_\alpha H}$ | | $J_{C_\alpha HC_\beta H}$ | | | |
|---|---|---|---|---|---|---|
| | D-Valine | L-Valine | D-Valine | L-Valine | L-Lactate | D-Hyiv[b] |
| | 8.0 | 6.2 | 9.6 | 10.1 | 7.0 | 3.4 |
| LiTNC | 6.0 | | 10.5 | | 7.0 | 3.4 |
| NaTNC,NaSCN | 6.0 | | 10.3 | | 7.1 | 3.4 |
| (NaTPB) | (5.0) | | | | | |
| KTNC, KCl, KSCN | 4.8 | | 10.8 | | 6.8 | 3.4 |
| RbCl | 5.0 | | 10.2 | 10.8 | 6.8 | 3.4 |
| CsCl | 5.0 | | 10.3 | 10.2 | 7.0 | 3.4 |
| $NH_4TNC$ | 4.8 | | 10.6 | | 7.1 | 3.4 |

[a] In Hz (±0.2). [b] D-Hyiv, D-hydroxyisovalerate.

ions in biological processes, complexes of these cations were studied the most thoroughly.

## Experimental Section

*Materials.* Valinomycin was purchased from Cal biochem, and as with other reagents (unless specified) was used without further purification.

The metal ion complexes were formed in either of two ways. One procedure involved dissolving valinomycin and excess salt in Spectroscopic grade methanol and evaporating the solution to dryness under a stream of dry nitrogen. The residue was then dissolved in deuterated chloroform (Merck). The other procedure for forming the complexes allowed crystals of the appropriate salt to stand in contact overnight with deuterated chloroform solutions of valinomycin. For the $TNC^-$ salts, which are themselves insoluble in $CDCl_3$, a bright yellow-orange color appeared in the $CDCl_3$–valinomycin solutions almost immediately upon contact with the TNC salt crystals. The color of these solutions, due to the anion absorption bands at ~355 and 450 nm, provided a convenient way to determine the concentration and stoichiometery of the valinomycin–metal ion complex. The molar extinction coefficients, $\epsilon$, in $CHCl_3$ are 1.189 (±0.003) $\times 10^4$ ($\lambda_{max}$ 355 nm) for the NaTNC–VAL complex and 1.275 (±0.005) $\times 10^4$ ($\lambda_{max}$ 360 nm) for the KTNC complex. In hexane, $\epsilon_{NaTNC}$ is 0.974 (±0.005) $\times 10^4$ ($\lambda_{max}$ 340 nm) and $\epsilon_{KTNC}$ is 1.148 (±0.008) $\times 10^4$ ($\lambda_{max}$ 355 nm).

The TNC salts were prepared by mixing molar equivalents of the free acid (Eastman) and the appropriate bases in methanol or water. Crystals formed after evaporation of the solvent were collected, washed with chloroform to remove any free acid, and then recrystallized from methanol.

Trace amounts of water and HDO were removed from the deuterated acetone (Merck) by vacuum distillation and storage of the solvent over molecular sieves.

*Methods.* Proton NMR spectra were recorded on both commercial (Bruker-90 MHz; Varian-100 MHz) and laboratory (MPC-250 MHz) spectrometers (Dadok et al., 1970). All the spectrometers were operated in frequency-swept, field-locked modes, using the resonance line of dissolved tetramethylsilane ($Me_4Si$) for the lock signal and frequency reference. Most spectra were recorded at least four times and the chemical shifts and coupling constants reported below are the average values for three separate experiments. The ambient sample temperatures were ca. 25° in the 90- and 250-MHz probes and 30° in the 100-MHz probe. Variable temperature experiments utilized the temperature control units provided with the spectrometers. The temperature settings were calibrated by measuring the chemical shifts of the hydroxyl protons of methanol or glycerol and comparing these with standard curves.

Carbon-13 NMR spectra were obtained at 22.6 MHz by the Fourier transform technique (Farrar and Becker, 1971). Proton noise decoupling was utilized to simplify the spectra and to improve the signal-to-noise characteristics via the Overhauser enhancement mechanism (Kuhlmann and Grant, 1968).

Optical absorption measurements were made with a Zeiss Model PMQ-11 spectrophotometer using 1-cm path length quartz cuvets. Circular dichroism was measured using a Sproul Scientific SS-20 CD modification of the Durrum-JASCO J-15.

The solubilities of the Na and KTNC complexes of valinomycin in hexane were determined by measuring the optical densities at the absorption maximum in hexane solutions saturated with the Na- or KTNC–valinomycin. The solubility of free valinomycin was measured similarly after converting a known aliquot of the valinomycin-saturated hexane solution to the NaTNC complex. Crystals of the appropriate compounds were allowed to equilibrate in stoppered vials for approximately 1 week.

The titration of valinomycin in acetone-$d_6$ with K- and NaTNC was performed by adding Na- or K-TNC from a $10^{-2}$ *M* (acetone-$d_6$) stock solution to 0.4 ml of $5.7 \times 10^{-3}$ *M* valinomycin in successive amounts of 25 $\mu$l. The concentrations of the reactants were adjusted for dilution.

## Results

*Proton NMR Spectra in Low Polarity Solvents.* The chemical shifts and spin–spin constants associated with the backbone protons of valinomycin and several monovalent cation complexes in deuterated chloroform solutions are given in Tables I and II. Also given in Table I are the tem-

Table III: Proton Chemical Shifts of Valinomycin and Its K and Na Complexes in Hexane and $CCl_4$.[a,b]

| Solvent | Salt | NH | | $C_\alpha H$ | | | |
|---|---|---|---|---|---|---|---|
| | | D-Valine | L-Valine | D-Valine | L-Valine | L-Lactate | D-Hyiv[c] |
| Hexane | | 8.15 | 8.06 | 3.98 | 3.91 | 5.35 | 5.10 |
| $CCl_4$ | | 7.90 | 7.78 | 3.95 | 3.88 | 5.21 | 4.95 |
| Hexane | NaTNC | 8.35 | 8.30 | 3.81 | | 5.31 | 5.06 |
| $CCl_4$ | NaTNC | 8.16 | 8.11 | 3.82 | 3.72 | 5.21 | 4.97 |
| $CCl_4$ | KTNC | 8.13 | | 3.74 | 3.70 | 5.17 | 4.72 |

[a] In ppm (±0.02) from TMS; spectra in hexane were recorded at 90 MHz, in $CCl_4$ at 100 MHz. [b] $2-10 \times 10^{-3}$ *M*. [c] D-Hyiv, D-hydroxyisovalerate.

Table IV: Proton Chemical Shifts[a] and Vicinal Coupling Constants[b] for Valinomycin and Its Na and K Complexes in Acetone.

| Salt | NH | | $C_\alpha H$ | | | |
|---|---|---|---|---|---|---|
| | D-Valine | L-Valine | D-Valine | L-Valine | Lactate | Hyiv[d] |
| | 7.64 | 7.90 | 4.36 | 4.16 | 5.39 | 5.02 |
| NaTNC[c] | 7.83 | 8.03 | 4.25 | 4.13 | 5.32 | 4.97 |
| KTNC[c] | 8.47 | 8.40 | 3.94 | 3.90 | 5.07 | 4.75 |
| Salt | $J_{NHC_\alpha H}$ | | $J_{C_\alpha HC_\beta H}$ | | | |
| | 8.5 | 7.0 | 8.0 | 9.5 | 6.8 | 3.5 |
| NaTNC[c] | 7.5 | 6.5 | 8.5 | 9.0 | 7.0 | 3.7 |
| KTNC[c] | 5.0 | | 11.0 | | 7.3 | 3.5 |

[a] In ppm (±0.02) from $Me_4Si$. [b] In Hz (±0.2). [c] Chemical shifts and coupling constants represent limiting values for increasing salt concentration (see text). [d] Hyiv, hydroxyisovalerate.

perature coefficients for the amide proton chemical shifts of free valinomycin and its Na and K complexes. The line assignments were made by comparing relative intensities, splitting patterns, and by comparison with the results of other investigators (Shemyakin et al., 1969; Ivanov et al., 1971). In some cases double resonance techniques were used to identify the appropriate multiplets of the valine $C_\alpha$-H pairs.[2]

Similar chemical shift data for valinomycin as well as its Na and K complexes in the nonpolar solvents, $CCl_4$ and hexane, are given in Table III. The NMR spectrum of the K complex could not be recorded in hexane owing to its low solubility.[3] As in $CDCl_3$, the $NHC_\alpha H$ vicinal coupling constants for free valinomycin are reduced from 8 and 7 Hz to 5 Hz upon complex formation. For the other sets of vicinal coupling constants no significant differences are observed between the cation complexes and free valinomycin.

*Proton NMR Spectra in Acetone.* Proton chemical shifts and coupling constants are given in Table IV for valinomycin, Na-valinomycin, and K-valinomycin dissolved in acetone-$d_6$ at 25°. The values of the NMR parameters for the complexes are those obtained in the limit of increasing concentrations of Na or K ions. This concentration dependence is a consequence of the exchange of cations between complexed and free valinomycin. It can be used to estimate the cation-association constants as well as the rates of cation-complex dissociation.

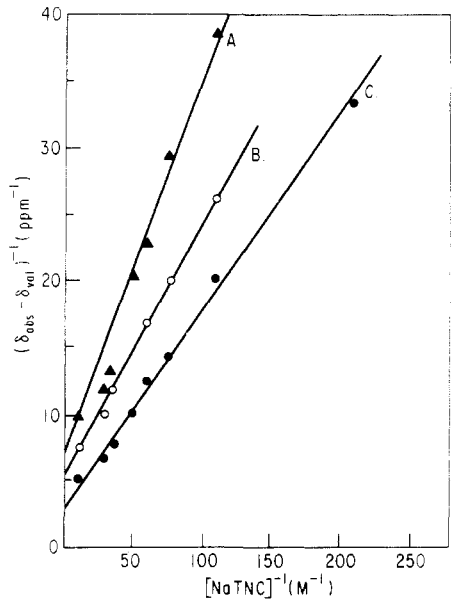


FIGURE 1: The effect of NaTNC on the chemical shifts of the (A) L-valine $C_\alpha$-H; (B) D-valine N-H; and (C) L-valine N-H protons of valinomycin in acetone-$d_6$. The data are plotted according to eq 1′ of the text. The concentration of valinomycin is $5.7 \times 10^{-3}$ *M* and the spectra were recorded at 250 MHz and a temperature of 25°. It is assumed that [NaTNC] ~ $[NaTNC]_{tot}$ (i.e., [NaTNC] ≫ [NaVAL]) and that ion pairing is negligible.

When NaTNC is added to an acetone solution of valinomycin, only a single set of sharp resonance lines is observed and the frequencies depend upon the concentration of the added salt. This is the "fast exchange" condition (Pople, et al., 1959) where the reciprocals of the lifetimes for the free and complexed species, $(\tau_{VAL})^{-1}$ and $(\tau_{NaVAL})^{-1}$, are large compared to the absolute frequency difference, $|\nu^m_{VAL} - \nu^m_{NaVAL}|$ between the resonances of any proton group, m, in the two forms. When "fast exchange" occurs, the position of the observed line may be expressed as:

$$\nu_{obsd} = (1 - X_{NaVAL})\nu_{VAL} + X_{NaVAL}\nu_{NaVAL} \quad (1)$$

where $X_{NaVAL}$ and $1 - X_{NaVAL}$ are the fractions of complexed and free valinomycin molecules. In the limit, $X_{NaVAL} \rightarrow 1$; $\nu_{obsd} \rightarrow \nu_{NaVAL}$. These limiting values are the ones given in Table IV.

Since the fractions of the free and complexed species may be expressed in terms of the association constant and the NaTNC concentration, i.e., $(X_{NaVAL})/(1 - X_{NaVAL}) = K_{assoc}[NaTNC]$, eq 1 may be cast in the following form:

$$(\nu_{obsd} - \nu_{VAL})^{-1} = (\nu_{NaVAL} - \nu_{VAL})^{-1}(1 + K_{assoc}[NaTNC]^{-1}) \quad (1')$$

By plotting the left-hand side of (1′) as a function of $[NaTNC]^{-1}$, the slope and intercept yield two quantities from which $K_{assoc}$ and $\nu_{NaTNC}$ may be determined. Such graphs for the amide and valine $C_\alpha H$ lines are shown in Figure 1. The association constant estimated in this way is

[2] Double resonance ("tickling") was also used to verify that the D- and L-valine $C_\alpha H$-$C_\beta H$ vicinal coupling constants in these compounds are 10–11 Hz. This was later confirmed by analysis of spectra recorded at 250 MHz. The magnitude of this coupling constant is of some importance in the structural analysis and there is some confusion in the literature as to the correct value. This is understandable owing to a chemical shift between the D and L $C_\alpha$ protons of 10 Hz at 100 MHz, and an infortuitous overlap of lines from the separate multipletes. Broadened lines further compound the difficulty. For references and a more detailed discussion see Urry and Kumar (1974).

[3] At 25° the maximum solubilities of free VAL, NaTNC-VAL and KTNC-VAL were found to be $1.4 \times 10^{-2}$, $2.5 \times 10^{-3}$, and $1.2 \times 10^{-4}$ *M*, respectively.

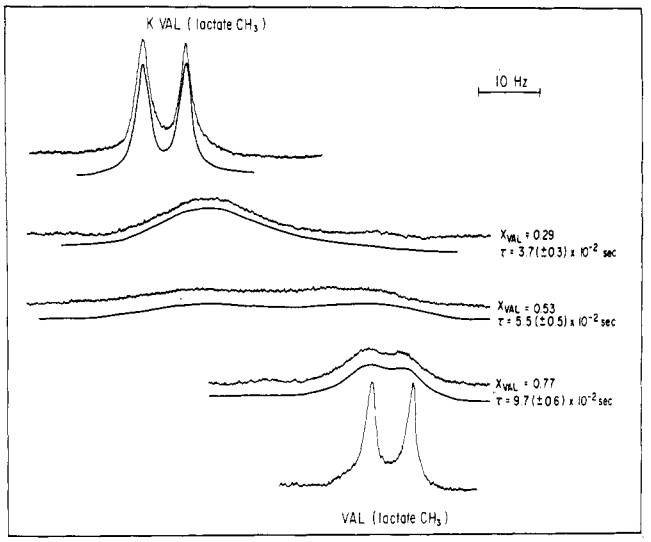


FIGURE 2: The effect of KTNC on the lactate methyl proton resonance of valinomycin in acetone-$d_6$. The spectra were recorded at 250 MHz, 25°, and with a total valinomycin concentration of $5.7 \times 10^{-3}$ *M*. From bottom to top, the spectra correspond to salt concentrations of: no salt; $1.4 \times 10^{-3}$ *M*; $2.8 \times 10^{-3}$ *M*; $4.2 \times 10^{-3}$ *M*; and $7 \times 10^{-3}$ *M* KTNC, respectively. The smooth curves directly below each experimental trace were computed according to the procedure referred to in the text. The values of $X_{VAL}$ and $\tau$ which give the best agreement between the computed and observed spectra are listed to the right of the figure.

25 (±5) $M^{-1}$. A lower limit on the exchange rate also may be approximated from the largest chemical shift difference, i.e., $(\tau_{NaVAL})^{-1} \gg |\nu^{NH}_{NaVAL} - \nu^{NH}_{VAL}| \sim 80$ sec$^{-1}$.

For KTNC, $(\tau_{KVAL})^{-1}$ and $(\tau_{VAL})^{-1}$ are comparable in magnitude to the frequency difference between the proton resonances in the free and uncomplexed molecules. Therefore, the spectra of the mixture is best described by a formula originally derived by Gutowsky et al. (1953). In this formulation, assuming that the chemical shifts and line widths for VAL and K-VAL can be determined independently, the spectrum is a function of $(X_{KVAL} - X_{VAL})$ and an exchange parameter:

$$\tau \equiv X_{KVAL}\tau_{VAL} = X_{VAL}\tau_{KVAL} \qquad (2)$$

By fitting the calculated to the observed spectra, $\tau$, $X_{KVAL}$ and $X_{VAL}$ are determined uniquely. Figure 2 shows the effects of cation dissociation on the lactate methyl resonances at different molar ratios of KVAL and VAL.[4] Here the mole fractions, $X_{KVAL}$ and $X_{VAL}$, were adjusted by adding known amounts of KTNC to $5.7 \times 10^{-3}$ *M* solutions of VAL in acetone-$d_6$. The doublet structure is due to the 7-Hz coupling to the $C_\alpha$ proton.[5] The smooth curves immediately below the experimental lines are those computed from the complete exchange-modified line-shape formula. The values of $\tau$ and $X_{VAL}$ giving the best visual fit to the observed spectra are indicated to the right of the curves. From these data the apparent average lifetime of the K complex, $\tau_{KVAL}$, can be calculated (eq 2) for each value of $\tau$ and $X_{VAL}$. For $X_{VAL} = 0.77$, 0.53, and 0.29; $\tau_{KVAL} = 0.97\ (\pm 0.06) \times 10^{-1}$; $1.04\ (\pm 0.06) \times 10^{-1}$; and $1.3\ (\pm 0.1) \times 10^{-1}$ sec, respectively. Within experimental error, $\tau_{KVAL}$ is independent of the concentration of KVAL and VAL. A *lower* limit of $10^4$ $M^{-1}$ can also be estimated[6] for the equilibrium association constant, $K_{assoc} \equiv$ [KVAL]/[K][VAL].

Table V: Carbon-13 Chemical Shifts for Valinomycin and Its NaSCN and KSCN Complexes in $CDCl_3$.[a,b]

| Carbon Atom | Valinomycin | NaSCN Complex | KSCN Complex |
|---|---|---|---|
| C=O | 172.8[e] | 174.1[e] | 176.5[e] |
| | 172.1[f] | 173.6[f] | 175.8[e] |
| | 171.2[e] | 172.2[f] | 173.3[f] |
| | 170.4[f] | | 171.8[f] |
| $C_\alpha$–O | 79.0 | 79.0 | 80.0[c] |
| | 70.7 | 71.0 | 71.5[d] |
| $C_\alpha$–N | 60.7 | 61.8 | 62.1 |
| | 59.2 | 61.4 | 61.8 |
| $C_\beta$H | 30.5 | 30.6 | 30.5 |
| | 28.7 | 28.5 (2 lines) | 28.7 |
| $CH_3$ | 19.7 | 20.0 (2 lines) | 20.4 |
| | 19.4 | 19.4 | 19.9 |
| | 17.2 | 19.1 | 19.5 |
| | 16.7 | 18.1 | 19.2 |
| | | 16.8 | 17.7 |
| | | | 17.0 |

[a] Chemical shifts are given relative to $Me_4Si$ (±0.2 ppm). [b] Concentration ~ 0.1 *M*. [c] Hydroxyisovalerate. [d] Lactate. [e] Ester linked. [f] Amide linked.

*Carbon-13 NMR Spectra in CDCl$_3$.* The $^{13}C$ chemical shifts for valinomycin and its Na- and KSCN complexes (measured relative to $Me_4Si$ in $CDCl_3$) are given in Table V. The assignments of the resonances for carbon nuclei of the $C_\alpha$–N, $C_\alpha$–O, $C_\beta$, and $CH_3$ groups are based upon the studies of Ohnishi et al. (1972), where the shifts measured for model compounds can be extrapolated to valinomycin in a straightforward way. The ester and amide carbonyl lines for free valinomycin were identified by comparison with the $^{13}C$ spectrum for this compound in cyclohexane as analyzed by Grell et al. (1973).[7] The carbonyl groups for the KSCN complex can be assigned readily and unambiguously because the interaction of the K ion with the ester carbonyl oxygens shifts the $^{13}C$ resonances of these groups 4.4–5.5 ppm downfield from their positions in the uncomplexed molecule. On the other hand, the amide carbonyls are shifted downfield only by 0.5–0.6 ppm. For the Na-valinomycin complex, the line at 172.2 ppm and one of the lines at 173.6 ppm are likewise assigned to amide carbonyl groups since their shifts are comparable with those for the KSCN complex. The line at 174.1 and the other at 173.6 ppm are thus

[4] Haynes et al. (1969) were the first to study the dissociation kinetics of KVAL via the lactate methyl resonance signal. Their study was limited, however, to the "slow exchange" condition and a single temperature.

[5] This is a weak coupling case ($JI_1I_2 \sim Jm_1m_2 \ll s|\nu_1 - \nu_2|$) where each component of the doublet may be treated as belonging to a separate molecule. That is there are two reactions:

$$\mathrm{KVAL}(m_2 = +\tfrac{1}{2}) \underset{k_{-1}}{} \mathrm{VAL}(m_2 = +\tfrac{1}{2}) + \mathrm{K}$$

and likewise for KVAL($m_2 = -\frac{1}{2}$) where $m_2 = \pm\frac{1}{2}$ refers to the spin state of the lactate $C_\alpha$ proton.

[6] Under the conditions used here the reaction is practically stochiometric and eq 2 can be rewritten as $\tau/\tau_{KVAL}(1 + [K]_{tot}/[VAL]_{tot}) \simeq (1 + K^{-1}_{assoc}[K]_{tot}/[VAL]^2_{tot}) +$ (terms of order $\sim K^{-1}_{assoc} \cdot [K]^2_{tot}/[VAL]^3_{tot}$ and $K^{-1}_{assoc}[K]_{tot}[VAL]^2_{tot}$). Within experimental error the left-hand side of the above equation is $1.0 \pm 0.1$ over the range of titration. Thus $K^{-1}_{assoc}[K]_{tot}/[VAL]^2_{tot} \leq 0.1$ or $K_{assoc} \geq 10^4$ $M^{-1}$.

[7] Attempts to confirm this analysis by off-resonance, proton decoupling were unsuccessful owing to the simultaneous weak coupling of the $C_\alpha$ protons to both carbonyl groups as well as the small Overhauser enhancement factors for the $^{13}C$ resonances of these groups.

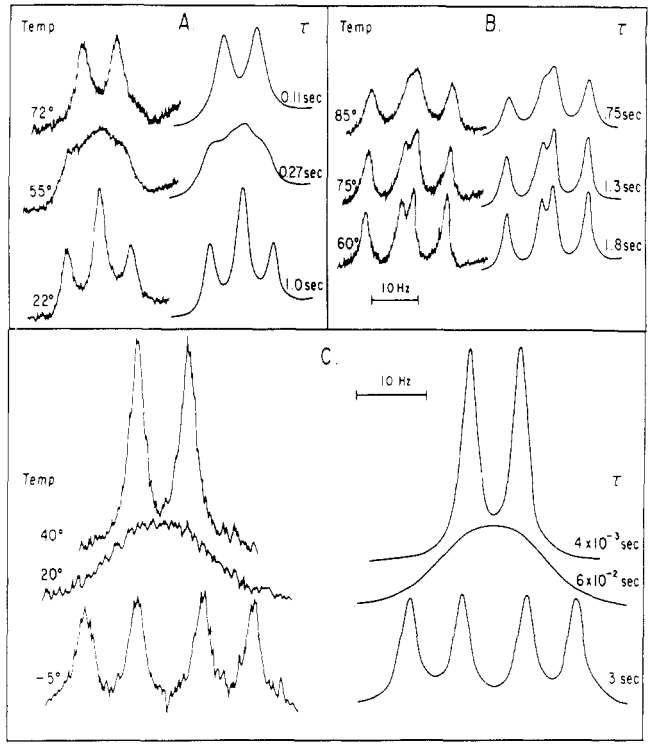


FIGURE 3: The effect of temperature on the lactate methyl proton resonance in approximately equimolar mixtures of valinomycin and its Na or K complexes: (A) $5.2 \times 10^{-3}$ *M* NaSCN-VAL + $4.8\ 10^{-3}$ *M* VAL in $CDCl_3$; (B) $4.5 \times 10^{-3}$ *M* KTNC-VAL + $5.5 \times 10^{-3}$ *M* VAL in $CDCl_3$; (C) $2.8 \times 10^{-3}$ *M* KTNC-VAL + $2.8 \times 10^{-3}$ *M* VAL in acetone-$d_6$. Experimental spectra, recorded at the temperature indicated, are shown to the left of each figure. Computed spectra and the values found to give the best fit are shown to the right. Spectra in (A) and (B) were recorded at 100 MHz; (C) was recorded at 90 MHz.

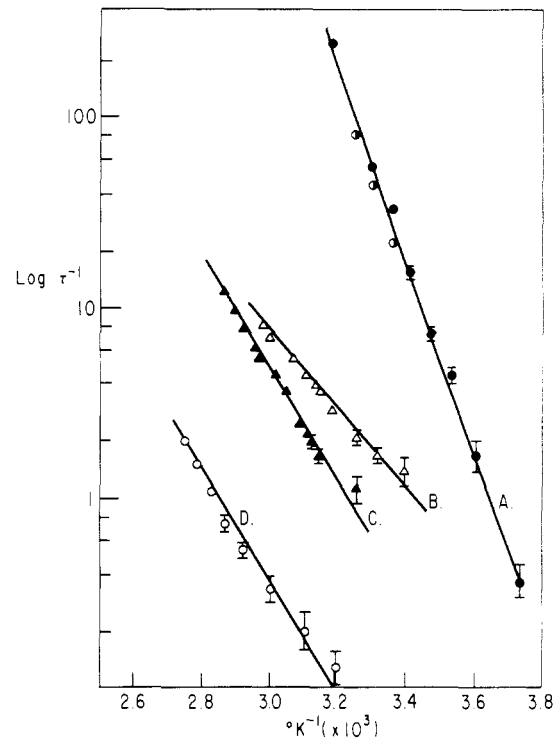


FIGURE 4: Arrhenius plots of $\tau^{-1}$ for mixtures of Na- or K-VAL in $CDCl_3$ or acetone-$d_6$: (A) (●) ~$2.8 \times 10^{-3}$ *M* KTNC-VAL + $2.8 \times 10^{-3}$ *M* VAL; (○) ~$1.4 \times 10^{-3}$ *M* KTNC-VAL + $4.2 \times 10^{-3}$ *M* VAL in acetone-$d_6$; (B) $5.7 \times 10^{-3}$ *M* NaTNC-VAL + $4.3 \times 10^{-3}$ *M* VAL in $CDCl_3$; (C) $5.2 \times 10^{-3}$ *M* NaSCN-VAL + $4.8 \times 10^{-3}$ *M* VAL in $CDCl_3$; (D) $4.5 \times 10^{-3}$ *M* KTNC-VAL + $5.5 \times 10^{-3}$ *M* VAL in $CDCl_3$. Error flags represent the uncertainty in the values of $\tau$ found to give the best visual fit between the computed and experimental spectra.

assigned to the ester-linked carbonyls of the Na-valinomycin complex.

*Temperature Dependence of the Average Lifetimes for the Cation Complexes.* By the same procedures that were used to estimate the average lifetime of the cation complex in the K ion titration of valinomycin, line-shape analysis of the spectra for equimolar mixtures of free valinomycin and the NaTNC-, NaSCN-, or KTNC-VAL complexes in $CDCl_3$ was carried out at different temperatures. The same experiments were also performed for acetone solutions of KTNC and VAL at 1:4 and 1:1 molar ratios of K to VAL.

With the exception of KTNC-VAL in $CDCl_3$, it was possible to vary the temperatures so that exchange could be followed at rates above and below the coalescence rate, i.e., $\tau^{-1} \equiv 2^{1/2}(\nu_{MVAL} - \nu VAL)$ (Pople et al., 1959). Again the spectra of the lactate methyl groups were used to monitor exchange (Haynes et al., 1969). Some typical spectra are shown in Figure 3. In a separate experiment it was also found that the line shape and, hence, the exchange rate for an equimolar mixture of VAL and NaTNC-VAL in $CDCl_3$ did not change over a tenfold dilution of the reactants.

Plots of ln $(\tau)^{-1}$ vs. $T^{-1}$ (°K) are shown in Figure 4 for different combinations of salts, solvent, and concentration. Within experimental accuracy, the curves are linear and show that kinetics follow a simple Arrenhius type of thermal activation:

$$(\tau)^{-1} = (\tau_0)^{-1} \exp(E_{act}/RT) \qquad (3)$$

From the slopes of the best linear curves through the data points in Figure 4, activation energies of 13.9 ± 0.4; 10.2 ± 0.3, and 13.0 ± 0.2 (in kcal $mol^{-1}$) are found, respectively, for 1:1 mixtures of valinomycin and its NaSCN, NaTNC, and KTNC complexes in $CDCl_3$. For the 1:4 and 1:1 mixtures of KTNC and VAL in acetone-$d_6$, the exchange reaction is found to have an activation energy of 29 ± 2 kcal $mol^{-1}$.

## Discussion

*Low Polarity Solvents; Proton Spectra.* For the most part, the proton NMR spectra of all the complexes in $CDCl_3$ are consistent with what is known about the conformation of the K-valinomycin complex from X-ray structural data (Pinkerton et al., 1969) as well as the NMR studies of others (Shemyakin et al., 1969; Ivanov et al., 1971). When the spectra of the complexes are examined in detail, however, distinct and significant differences are found between the Li and Na complexes on the one hand, and, on the other, the complexes of larger cations (K, Rb, Cs, $NH_4$, and Tl).

These differences are seen most clearly in the chemical shifts of the hydroxy acid $C_\alpha$ protons (Table I). In the LiTNC complex and all the Na complexes but one (NaTPB-VAL), the shifts of these lines are no different from those in free valinomycin. This finding is to be compared with that for the complexes with larger cations, where the $C_\alpha$ proton lines are shifted upfield by ~0.4 ppm. The difference is especially noteworthy in light of the spectrum of the NaTPB complex which is quite similar to that of the K, Rb, and Cs complexes. Likewise, the $NHC_\alpha H$ vicinal coupling constants for the LiTNC, NaBr, $SCN^-$, and

$TNC^-$ complexes, while smaller than in free valinomycin, are at least 1 Hz larger than those of the other complexes, including NaTPB.

The effect of the anion on the proton NMR spectra of the Na complexes is, therefore, quite clear and suggests that the conformation of the Na (and Li) complexes of smaller anions may be intermediate between that of free Val and the NaTPB complex or the K complexes. As the data in Tables I and II show the K complexes are themselves independent of the anion.

The proton NMR spectra of VAL and its Na or K complexes in hexane and $CCl_4$ (Table III) share most of the spectral properties of the compounds in $CDCl_3$ solutions. Again there is no change of the hydroxy acid $C_\alpha$ proton shifts for the NaTNC complex in either hexane or $CCl_4$. Unfortunately, the low solubility of the K-VAL complex in hexane precludes measurement of its spectra in this solvent. The spectra of the Na and K complexes may be compared in $CCl_4$, however. Although the spectral differences are not as marked as in chloroform, the spectra show, nonetheless, that differences involving the hydroxy acid $C_\alpha$ protons persist in $CCl_4$ as well. Moreover, the solubilities in hexane[3] clearly show that these two compounds have different physical properties and further support the notion that the Na and K complexes may have subtle, but distinct differences in their structures or conformation.

*Carbon-13 Spectra.* Differences between the Na- and K-valinomycin complexes are also apparent from their $^{13}C$ NMR spectra. As judged by the chemical shifts induced upon complex formation, it is clear that the Na ions like K interact directly with the valine (ester-linked) carbonyl groups of valinomycin. The induced shifts are smaller, however, and indicate that Na is less effective than K in polarizing the carbonyl groups, thus suggesting a weaker ion–ligand interaction. Likewise, the ion-induced shifts of atoms which are related by pseudoinversion symmetry suggest that the Na interacts asymmetrically with valinomycin. For example, the ester carbonyls of the K complex shift by 4.4 and 5.4 ppm, differing by ~20–23%. For the Na complex the shifts are 2.0 and 3.2 ppm and differ by ~38–60%. This asymmetry is also exhibited by $C_\alpha$–O carbon resonances which shift downfield by 1.0 and 0.8 ppm in the K complex; for the Na ion complex the corresponding lines shift by 0.0 and 0.3 ppm.

A weaker and asymmetric interaction of Na ions with valinomycin was also noted by Grell et al. (1973) in their $^{13}C$ NMR studies in $CH_3OH$. As discussed in the following section their finding can be related primarily to the ion-solvating and H-bonding properties of the solvent. In a low-polarity solvent such as chloroform, these features are (as are the structural differences indicated by the $^1H$ NMR spectra) rather consequences of different Na and K interactions with anions. The full discussion of this matter is deferred to the final section of this paper.

Independent experimental evidence for the asymmetric character of the Na complex as well as differences between Na- and K-VAL ion pairs in nonpolar solvents is provided, however, by CD spectra of Na- and KTNC-valinomycin in the region of the TNC absorption bands (Figure 5). Since the TNC ion is optically inactive, the obvious circular dichroism of the NaTNC complexes must be induced by some optically active center that is related to the valinomycin moiety. Clearly a greater asymmetry of the cation complex as well as closer contact between the complex and the anionic chromophore would be expected to enhance the induced Cotton transitions of the Na complex over those of the K complex.

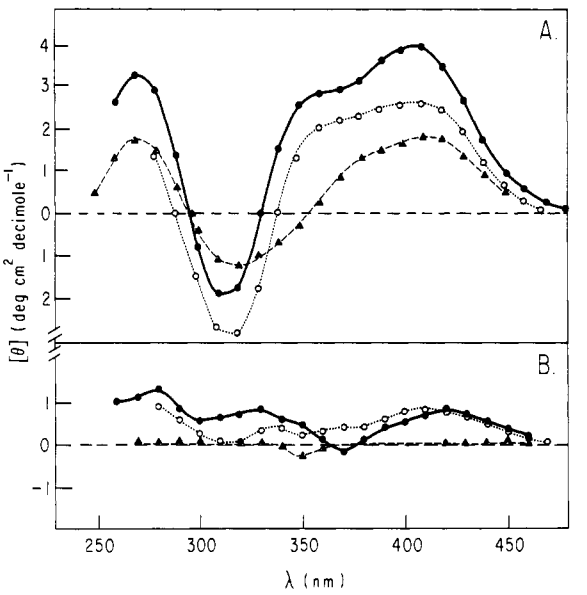


FIGURE 5: Partial circular dichroism spectra of (A) NaTNC-VAL and (B) KTNC-VAL in low polarity solvents. The scale on the ordinate is in units of molar ellipticity, $[\theta]$ (deg cm$^2$ dmol$^{-1}$). In these samples, only the TNC ion absorbs light in this region of the spectrum. (●) $6.6 \times 10^{-5}$ *M* Na- or K-VAL in hexane; (○) $2.5 \times 10^{-4}$ *M* Na- or K-VAL in $CCl_4$; (▲) $2.2 \times 10^{-4}$ *M* Na- or K-VAL in $CHCl_3$.

*Na and K Complexes in Acetone.* Judging by the proton NMR spectra in acetone as well as by studies of others in comparable solvents (e.g., $Me_2SO$) (Ivanov et al., 1969; Ohnishi and Urry, 1969; Shemyakin et al., 1969), free valinomycin is in the so-called B-type (open) conformation whereas the K-VAL complex has the same $A_2$ (bracelet-like) conformation it also has in $CDCl_3$. On the other hand, the spectrum of the Na complex differs not only in comparison with the K form but also in comparison with Na-VAL in $CDCl_3$. In fact the spectrum of the Na complex in acetone is close to that of free valinomycin. Nonetheless, the NaVAL spectrum approaches a limit that is different from free valinomycin thus indicating that Na forms a structurally distinct, although labile complex.

The estimated equilibrium binding and dissociation rate constants clearly demonstrate the specificity of valinomycin for K over Na ions in acetone. These data also show that the absolute stabilities of the complexes are reduced in this solvent as compared with chloroform. The latter surely reflects solvent stabilization of the reactants both with regard to the free ions Parker (1962) as well as the uncomplexed valinomycin molecules. Indeed, in the B-type conformation, free valinomycin has three exposed amide protons that are bonded to solvent molecules so that just half its ligands can coordinate effectively to cations (Ovchinnikov, 1972).

With regard to the selectivity, it is interesting to note that nonactin, another membrane-active, cation-selective cyclic antibiotic, has been found to bind Na and K ions with equally high affinities (~$7 \times 10^4$ $M^{-1}$) in acetone (Prestegard and Chan, 1969). In contrast, it is shown here that valinomycin retains a selectivity for K over Na. As discussed by Prestegard and Chan (1969), the difference in the free energy of interaction between the cation and the acetone carbonyl groups on the one hand, and the cation and nonactin ligands, on the other hand, is independent of the ionic size. This is clearly not the case for Na and K ions reacting

with valinomycin in acetone. An explanation for this may be that the electrostatic stabilization of the $Na^+$ via coordination to the valine carbonyls is limited by the conformational constraints imposed by the primary structure of valinomycin, whereas such constraints are not present in the primary structure of nonactin.

*Kinetic Studies.* The kinetics and the temperature dependence of Na and K interacting with valinomycin reveals a number of features concerning the reactions of these compounds and the way they are effected by the solvent. At the concentrations studied, the lifetimes, $\tau_{MVAL}$, for all the cation complexes are independent of the reactant concentrations. Thus unimolecular dissociation of the cations from valinomycin rather than cation exchange between loaded and unloaded valinomycin molecules determines the lifetime of the complexed state. At higher concentrations of free valinomycin, however, the exchange reaction may become more important (Haynes, 1972).

From the Arrhenius plots shown in Figure 4 it can be seen that the dissociation rate of NaTNC-VAL in $CDCl_3$ (curve B) is 20 times faster than KTNC-VAL (curve D). Although the activation energy for the dissociation of the Na complex is ~3 kcal $mol^{-1}$ smaller than for the K complex, the principal factor governing the difference in the rate constants is temperature independent and can be identified with the entropy of activation for the reactions. The lower activation energy for the Na complex is consistent with weaker Na ion–carbonyl group interactions as deduced from the $^{13}C$ NMR data. As discussed in the next section, the lower entropy (hence lower *free* energy) of activation for the NaTNC-VAL complex may also be understood in terms of the asymmetric structure of this complex.

The approximate twofold increase in the activation energy for the dissociation of the K complex in acetone (Figure 4) is also understandable in light of the structures of the complexed and free forms. As pointed out above, free valinomycin in acetone has a B-type conformation with three fewer intramolecular H-bonds than the folded, $A_2$-type conformation of the K complex. To go from the complex to the free form, thermal energy is required not only to remove the K ion from the dipolar fields of the carbonyl groups but also to break three of the intramolecular H-bonds and change bond angles in the depsipeptide backbond. If one assumes that 13 kcal/mol (taken from the activation energy in $CDCl_3$) is a measure of the electrostatic portion of the activation energy, the remaining 16 kcal/mol partitioned among three H-bonds gives a reasonable estimate for the energy of such bonds.

Finally it is interesting to speculate that the structures of the Na complexes (two of which according to their NMR spectra are intermediate between free valinomycin and the K complex) may well be quite similar in structure to the transient intermediates of K–valinomycin. Such intermediates have been proposed by Grell et al. (1972) from their relaxation-kinetic studies.

*Correlations between the Structural and Functional Properties.* From the results of the studies reported here, it is concluded that valinomycin complexes with Na ions (but not K ions) can exist in at least three distinct conformational states, depending upon the anion and solvent. These different structures or conformations for the Na–valinomycin complexes may be described in terms of the ligands in the coordination shell of the Na ion. In low polarity solvents where the Na complexes exist predominately as ion pairs, it is proposed that only three of the six available valine carbonyl groups of the depsipeptide are coordinated strongly to the Na ion when $Br^-$, $SCN^-$, or $TNC^-$ is the counterion. It is most likely that a fourth coordination site on the Na ion is occupied by one of these anions, thus giving the asymmetric character to the association between the Na ion and the depsipeptide ligands. For the larger, bulkier tetraphenylboron counterions, the stability of the ion pair is reduced and the Na ion can then occupy an octahedral coordination site located at the pseudoinversion center of the depsipeptide moiety. For this particular Na complex the conformation is much like that of the K complex, where the cation is, likewise, at the center of the molecule (Pinkerton et al., 1969).

These proposed association schemes are consistent with and indeed bring together a number of seemingly unrelated facts about differences between the properties of Na- and K–valinomycin in low-polarity solvents. They explain for example why the proton NMR spectra of the Na complexes depend upon the anion while the K complexes do not and why the spectrum of the NaTPB complex resembles those of the complexes with the larger cations. The greater solubility of the NaTNC complex in hexane may also be understood qualitatively in terms of the smaller dipole moment and lower electrostatic free energy that is associated with the smaller spacing between NaVAL–TNC ion pairs. Here no intervening atoms of the depsipeptide separate the charges as in the KVAL–TNC complex. The closer contact between ion pairs also accounts for the observation that the absorption spectrum (Davis and Tosteson, 1971) as well as the CD spectrum of $TNC^-$ is different when it serves as the counterion to NaVAL as compared with KVAL in relatively nonpolar solvents. Finally the lower free energy of activation for the dissociation of the NaVAL complexes in chloroform is also consistent with their asymmetric, intermediate structure. Qualitatively, in terms of a transition-state diagram (Jencks, 1969) the Na complexes are closer structurally to the activated transition state, further along the reaction pathway and thus have a smaller free energy barrier to climb before dissociating.

As judged by the proton NMR data, the Na–valinomycin complex in acetone (at concentrations where ion pairing is negligible (Davies, 1962)) exhibits both another structural compromise between the types of ligands in the coordination shell of the cation as well as the consequences of solvent interaction with the depsipeptide. Here, the Na ion is again partially coordinated to polar ligands of valinomycin and remains exposed to the solvent and anions. At the same time H-bonding between solvent molecules and the amide protons of the depsipeptide tend to maintain the Na complex in a conformation more like that of the free, uncomplexed molecule. By contrast the conformation of the K complex maintains the octahedral coordination of depsipeptide ligands such that the K ion is shielded from the solvent and anion.

The relationships between the structures of the Na and K complexes and the cation selectivity of valinomycin are seen clearly and consistently in the experimentally estimated cation binding equilibrium constants, as well as the rate constants and activation energies for cation dissociation. The structural basis for the cation selectivity of valinomycin is, however, a subtle one. It includes considerations about the way the ligands of the depsipeptide bind to the cation in competition with the anion or solvent molecules. Selectivity also involves how the energy of interaction of the cation with the depsipeptide ligands is weighed against the energy to overcome configurational constraints that are dictated by

the primary sequence or by solvent stabilization of valinomycin in a conformation which is less than optimal for the binding of cations.

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