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Location and ion-binding of membrane-associated valinomycin, a proton nuclear magnetic resonance study

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Valinomycin, incorporated in small unilamellar vesicles of perdeuterated dimyristoylphosphatidylcholine, reveals several well-resolved ^{1}H-NMR resonances. These resonances were used to examine the location, orientation and ion-binding of membrane-bound valinomycin. The order of affinity of membrane-bound valinomycin for cations is $Rb^+ > K^+ > Cs^+ > Ba^{2+}$, and binding is sensitive to surface charge. The exchange between bound and free forms is fast on the NMR time scale. The intrinsic binding constants, extrapolated to zero anion concentration, are similar to those determined in aqueous solution. Rb^+ and K^+ show 1:1 binding to valinomycin, whereas the stoichiometry of Cs^+ and Ba^{2+} is not certain. Paramagnetic chemical shift reagents and nitroxide spin label relaxation probes were used to study the location and orientation of valinomycin in the membrane. Despite relatively fast exchange of bound cations, the time average location of the cation-free form of valinomycin is deep within the bilayer under the conditions of these experiments. Upon complexation to K^+, valinomycin moves closer to the interfacial region.

Introduction

Small molecules that facilitate diffusion of ions across biological membranes provide systems of such chemical simplicity as to reveal molecular or even atomic-level details of ion transport. Valinomycin is one such molecule that acts as an ionophore for alkali cations in biological membranes [1,2]. It is a cyclic depsipeptide found in several species of *Streptomyces* bacteria, which exhibits antimicrobial activity against many bacteria, yeasts and phytopathogenic fungi. It is a 12-membered ring composed of three residues each of L-lactic acid (L-lac), L-valine (L-val), D-hydroxyisovaleric acid (D-hyiv) and D-valine (D-val) with a molecular weight of 1111. Black lipid membrane and solvent partition studies have shown that valinomycin has a high selectivity for K^+ and Rb^+ over other alkali cations such as Na^+ or Li^+ [1,3,4]. Valinomycin transports ions across membranes via a carrier mechanism [5,6] with all transport steps (complexation, dissociation and movement across the membrane) occurring at comparable rates of about 10^4 s^{-1} [7]. The rate of valinomycin transport of K^+ across black lipid membranes is affected by both the thickness of

Abbreviations: DMPC, dimyristoylphosphatidylcholine; L-lac, L lactic acid; L-val, L-valine; D-hyiv, D-hydroxyisovaleric acid; D-val, D-valine.

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the bilayer [8] and the presence of surface charge on the membrane [9].

Most black lipid membrane data indicate a 1 : 1 valinomycin-K^+ complex. However, based on circular dichroic measurements, Ivanov [10] suggested that a stoichiometry of 1 : 2 for the K^+-valinomycin complex may exist in ethanol. This was termed the 'sandwich complex'. Furthermore, evidence based on NMR measurements was presented by Vishwanath and Easwaran [11] that the Ca^{2+} complex in acetonitrile is actually a 1 : 2 Ca^{2+}-valinomycin complex. The existence of these complexes suggests the posibility that ion translocation across the membrane could occur via a shuttling of the ion from one valinomycin molecule to another rather than movement of a single 1 : 1 complex across the membrane. It has not yet been determined whether these types of complex exist in vesicle membranes.

The location of valinomycin in the membrane has been studied previously by observing the effect of added valinomycin on the phospholipid in the membrane. Finer et al. [12] concluded that valinomycin interacts strongly with the fatty acyl chains of the phospholipid, based on broader ^{1}H-NMR resonances from the phospholipid fatty acyl chains in vesicles prepared in the presence of valinomycin. However, it is possible that acyl chain motion is affected without penetration of valinomycin into the interior of the bilayer. Hsu and Chan [13] reported that valinomycin interacts mainly with the headgroup of dipalmitoylphosphatidylcholine multilamellar vesicles based on the observation of a broader choline methyl resonance in vesicles prepared in the presence of valinomycin. For dimyristoylphosphatidylcholine, these investigators proposed that valinomycin interacts with the acyl chains as well as the headgroup, because of the observation of a decrease in the lipid phase transition temperature. Grell et al. [14] proposed that uncomplexed valinomycin is buried in the hydrophobic part of the bilayer, based on the similarity of the circular dichroism of valinomycin in phospholipid vesicles as compared to hydrocarbon solvents.

The synthesis of perdeuterated phospholipids has facilitated the direct observation of ^{1}H-NMR resonances of small molecules such as valinomycin incorporated into membranes [15–18]. In an earlier publication [19], we reported that in a lipid bilayer valinomycin appears to assume the 'A_2 bracelet' conformation which had been found in nonpolar solvents [20], and that the Rb^+-bound form of valinomycin in lipid bilayers assumes the conformation which had been found in all solvents and in the crystal form [21–23].

Though it is known that valinomycin acts by a carrier mechanism, i.e., involving diffusion of a valinomycin-cation complex in the membrane rather than diffusion of a cation through a valinomycin pore, the details of events actually occurring in the membrane are still not completely clear. Ion binding and motion of the molecule could be influenced strongly by the environment in the membrane. Conversely, the average location of valinomycin could be influenced by the binding of cations. In view of the differing results reported for the location of valinomycin in a bilayer and considering the usefulness of perdeuterated phospholipids for ^{1}H-NMR studies of small membrane-bound molecules, it was of great interest to try to determine the time average location of membrane-bound valinomycin, both in its free and ion-complexed forms. Furthermore, the stoichiometry of the ion-binding reaction and the affinity of various ions for membrane-bound valinomycin are important aspects of the ion-translocation mechanism which can not be determined directly in bulk solvent systems. The accessibility of these parameters by ^{1}H-NMR studies of the membrane-bound system allowed us to perform experiments to determine the orientation and location of the valinomycin molecule in the bilayer in its free and ion-complexed forms, as well as to determine the relative binding affinity of various cations for membrane-bound valinomycin.

Materials and Methods

Valinomycin was obtained from Sigma and used without further purification. Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti. The perdeuterated lipid, DMPC-d_{72} (91–99% labeled in all positions), was synthesized according to Kingsley and Feigenson [17] as modified by Meers and Feigenson [18]. All DMPC was greater than 99% pure, as determined by thin-layer chro-

matography before and after sonication. KSCN (99.9%) and NaSCN (99.9%) were from Fisher. CsSCN (98%), RbSCN (99%) and $Ba(SCN)_2$ (99%) were from Pfaltz and Bauer. Tetrabromophenolphthalein ethyl ester (99%) was from Eastman. Thin-layer chromatography was performed on Adsorbosil 5-P plates from Applied Science. Molecular exclusion chromatography was performed on columns of Sepharose 4B-CL from Pharmacia. ^2H_2O was from Aldrich. Water was purified on a Milli-Q system from Waters.

Valinomycin association with vesicles

The extent of incorporation of valinomycin into small unilamellar vesicles and the integrity of the vesicles were determined using gel filtration chromatography together with a valinomycin assay and a phosphate assay for phospholipid. Gel filtration separated vesicles according to size as well as from the original aqueous medium. For Sepharose column chromatography, 0.6 ml of vesicles made from 20 mg/ml DMPC sonicated in 50 mM sodium phosphate buffer at pH 7, in the presence or absence of 3 M KSCN, were loaded onto a column with a void volume of 20 ml. Void and included volumes were measured before vesicles were run, using Blue dextran and Chlorophenol red, respectively.

Valinomycin was assayed using an elaboration of the technique developed by Hyman [24]. The assay was chosen because it depends on fidelity of the potassium binding activity of valinomycin under the conditions of the experiments. In the absence of phospholipid the following assay procedure was used: 1 ml of 2 M KCl and 1 ml of 0.1% tetrabromophenolphthalein ethyl ester in chloroform were added to dry valinomycin in a small test tube, vortexed vigorously and the phases then allowed to separate. The lower, chloroform phase, was drawn up into a polypropylene syringe or pipettor tip, placed in a cuvette, and the absorbance at 616 nm was measured.

Because of phospholipid interference in the valinomycin assay, valinomycin must be extracted from samples containing phospholipid. To this end, aqueous dispersions of phospholipid containing valinomycin were frozen and lyophilized. The dry material was then vortexed with 1 ml of acetone and put on ice. After 5 min the tubes were centrifuged for 5 min at approx. $1000 \times g$. 800 μl of the acetone solution was withdrawn and transferred to a clean tube. This extraction was repeated twice. Acetone was removed by evaporation under a stream of nitrogen gas. The samples were further dried under high vacuum for several hours before assay. These samples were then treated as were the dry valinomycin samples above.

Total phospholipid was assayed by the method of Bartlett [25] as modified by Morrison [26].

Sample preparation

Samples for NMR were prepared by mixing the appropriate amount of a chloroform solution of valinomycin (3 mol% of the phospholipid concentration) and a chloroform solution of the phospholipid in a 10 ml Corex test tube and removing the solvent first under a stream of nitrogen, and then under high vacuum for at least 6 h. ^2H_2O or a ^2H_2O buffer was then added, the tube was sealed under argon and sonicated in a bath-type sonicator to clarity (approx. 30 min). For spin-labeled samples the sonication time was reduced somewhat to avoid degradation, and samples were checked by ESR for preservation of the spin label.

In order properly to compare the behavior of different cations at high concentrations, the measured ion concentrations were converted into ion activities. Activity coefficients for KSCN and NaSCN were taken from published data [27] or extrapolated from published data. Those for RbSCN and CsSCN were estimated based on comparisons to similar Rb^+ and Cs^+ salts and on the relationship to K^+ salts using KSCN as an adjustable model set of activity coefficients.

$Ba(SCN)_2$ activities were not estimated because of the lack of suitable data. Lipid samples used to measure binding isotherms were sonicated in 2.25 M activity NaSCN. Salts titrated into these samples were also at an activity of approx. 2.25 M. Activity coefficients of 0.52 for Rb^+ and K^+, 0.44 for Cs^+, and 0.80 for Na^+ were used at these concentrations.

NMR experiments

NMR experiments were performed on a Bruker WM-300 spectrometer operating at 300.12 MHz for protons. The sample temperature was regulated at 323 K unless otherwise specified. Chem-

ical shifts were measured relative to phospholipid methyl or methylene peaks, but reported relative to sodium 2,2-dimethyl-2-silapentane 5-sulfonate. The spin-lattice relaxation time, T_1, was measured by the inversion-recovery method [28]. In all T_1 experiments the incrementation of the recovery interval through the experiment was nonmonotonic in order to avoid systematic error. For particularly long experiments, the pulse sequence was programmed to change to a new recovery interval every four transients, and the data from each recovery interval were saved in a separate data file. Peak heights were used as a measure of the magnetization at each recovery time.

Results

In order to determine the partitioning of valinomycin between the phospholipid membrane and aqueous solution under the conditions of our experiments, the ratio of valinomycin to phospholipid was determined for the fractions from a Sepharose column loaded with valinomycin-containing phospholipid dispersions. In all samples examined, the ratio was relatively constant across the column profile, no free valinomycin was detected in the void volume, and 90% ± 20% of the valinomycin in the sample was accounted for in the lipid-associated fractions. Therefore, the valinomycin ^{1}H-NMR signals observed in our experiments are solely from membrane-associated valinomycin. Furthermore, the Sepharose column elution profile and the linewidths of ^{1}H-NMR resonances from vesicles sonicated in SCN^- salts were similar to those from vesicles in lower concentrations of Cl^- salts, indicating intact small unilamellar vesicles. Elution profiles from vesicles with or without valinomycin were nearly identical.

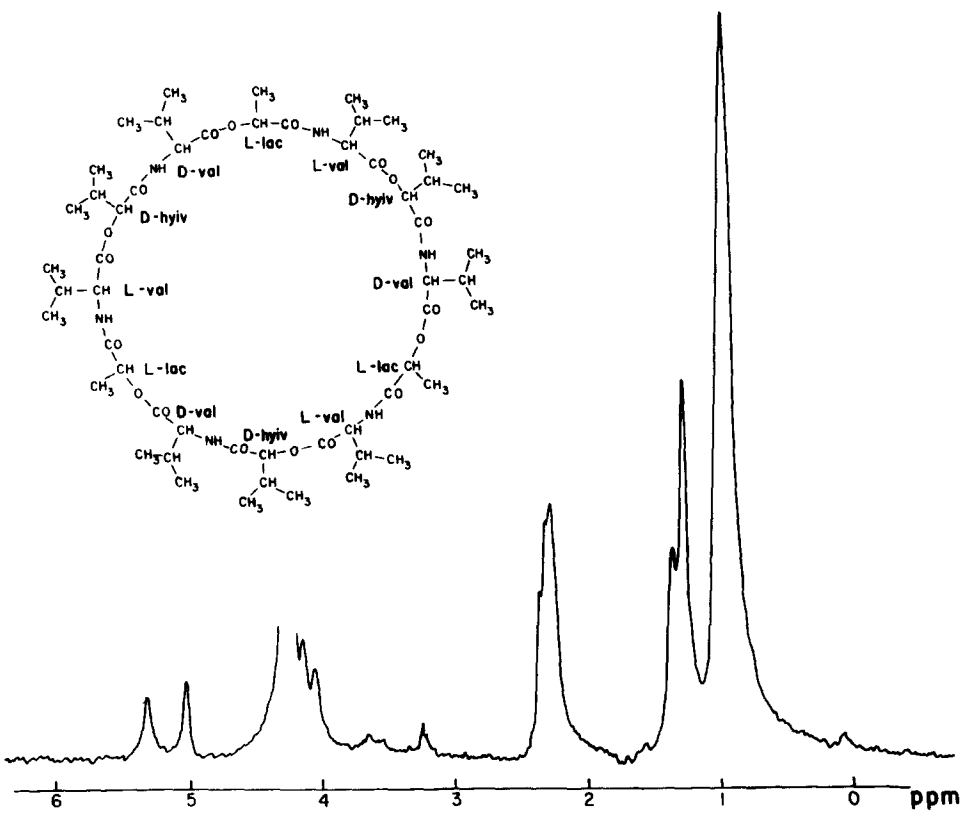

Fig. 1. 300 MHz ^{1}H-NMR spectrum of valinomycin in DMPC-d_{72} vesicles. Assignments are as follows: 5.34 ppm, L-lac α CH; 5.04 ppm, D-hyiv α CH; 4.3 ppm, H^2HO; 4.16 ppm, D-val α CH; 4.03 ppm, L-val α CH; 2.32 ppm, β CH of D- and L-valine and D-hyiv plus residual lipid acyl $C^2_\alpha HH$; 1.55 ppm, residual lipid acyl $C^2_\beta HH$; 1.36 ppm, L-lac CH_3; 1.27 ppm, residual lipid acyl C^2HH_n; 0.96 ppm, D- and L-val and D-hyiv CH_3. Experiments were performed at 50 °C with 10 mM total DMPC-d_{72} and 3 mol% valinomycin.

Fig. 1 shows the ^{1}H-NMR spectrum of valinomycin in sonicated vesicles of DMPC. As reported previously [19], resonances from the α protons of L-lac and D-hyiv at 5.34 and 5.04 ppm, respectively, are well resolved. The peaks at 4.16 and 4.03 ppm are from the D- and L-valine α protons, respectively. At 2.32, 1.36 and 0.96 ppm are the resonances from protons on β carbons and methyl carbons of valinomycin residues. Residual phospholipid resonances from the fatty acyl chains appear at 2.32, 1.55 and 1.27 ppm. The absence of a background resonance at approx. 5.25 ppm from the proton on the 2 carbon of the glycerol moiety of phosphatidylcholine is particularly useful. Elimination of this peak by deuteration results in well-resolved resonances from the α protons of D-hyiv and L-lac, thereby making possible the experiments described below. The reduction of the large signal from the rest of the phospholipid protons was also important in decreasing the dynamic range required for the data acquisition so that high gain could be used without receiver saturation.

Ion-binding

Since the cation-bound form of valinomycin has a different set of chemical shifts than the free form, it was possible to titrate the chemical shifts of several valinomycin resonances as an indication of cation-binding [19]. Fig. 2A shows isotherms of K^+ binding to valinomycin as monitored by the L-lac α, D-hyiv α and L-val α proton resonances. Each resonance shifts as a single peak up to a shift of as much as 150 Hz, indicating fast exchange between bound and free forms, on this time scale. For each resonance, data were obtained up to about 90% of the extrapolated maximum shift. The binding data were treated using the definition for the binding constant and the relationship [29]:

$$\Delta = ([\text{val}\cdot\text{cation}_n]/[\text{val}]_{\text{total}})\cdot\Delta_{\max} \quad (1)$$

where Δ is the observed change in chemical shift at a given cation concentration, $[\text{val}\cdot\text{cation}_n]$ the concentration of the cation-complexed form of valinomycin, n the ratio of cation to valinomycin in the bound complex, $[\text{val}]_{\text{total}}$ the total concentration of both free and complexed valinomycin, $\Delta_{\max}$ the maximum observed change in chemical shift. With the assumptions that the concentration of cation bound to valinomycin is negligible compared to the total concentration of the cation and that there is only one class of binding sites, we obtain:

$$\Delta/a_t^n = \Delta_{\max}K_{\text{app}} - (\Delta)K_{\text{app}} \quad (2)$$

where a_t is the total cation activity; K_{app} is the apparent binding constant, obtained by using the

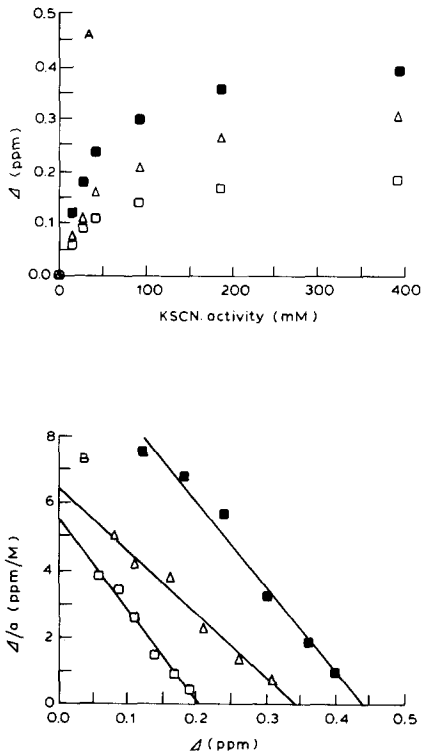

Fig. 2. (A) Binding isotherm of K^+ to valinomycin; change in chemical shift, Δ, vs. K^+ concentration. The binding of K^+ was monitored by the change in the chemical shifts of valinomycin resonances as K^+ concentration was increased. SCN^- concentration was held constant with NaSCN throughout the titration as described in Materials and Methods. The curves represent: □, L-lac α CH; ■, D-hyiv α CH; △, L-val α CH. (B) Plot of K^+ binding data, as described in the text, where Δ is the change in chemical shift and a is the activity of K^+. The straight lines are fits to the data by linear regression analysis. The binding constant is given by the negative of the slope of the plots. The maximum shifts are given by the x intercept. The curves represent: □, L-lac α CH; ■, D-hyiv α CH; △, L-val α CH; with binding constants derived from the slopes of 27.2 M^{-1}, 24.9 M^{-1} and 18.8 M^{-1} respectively. Experiments were performed at 50°C with 10 mM total DMPC-d_{72} and 3 mol% valinomycin.

total cation concentration for the binding constant rather than the local concentration near the membrane-aqueous interface. The appropriate stoichiometry, n, is indicated by linear plots of Δ/a_t^n vs. Δ with intercepts at the abscissa which are equal to the maximum change in chemical shift, Δ_{max}, derived from extrapolation of the binding isotherms. In Fig. 2B these plots are shown for the K^+ data with $n = 1$. The data were fit by simple linear regression with linear correlation coefficients greater than 0.91. Linear plots with similar slopes were observed for all three resonances. The data extrapolate to values quite close to the maximum shifts observed in the binding isotherms. The same data were treated as a Hill plot [30] using the extrapolated Δ_{max} values, and were also fitted by linear regression (correlation coefficients greater than 0.94). The slopes of the Hill plots gave binding ratios very close to 1, as shown in Table I. A similar treatment of Rb^+ data is shown in Fig. 3 and Table I. Hence for both K^+ and Rb^+, the valinomycin/cation ratio is determined to be 1 : 1.

In contrast to Rb^+ and K^+, much lower affinity binding was observed for Cs^+. It was not possible to approach the maximum shift in binding isotherms, but the linear fits to the data shown in Fig. 4 intercept the abscissa at points significantly below the Δ_{max} values found for the K^+ and Rb^+ complexes. Hill plots, using the Δ_{max} values for Rb^+ and K^+, yielded a Cs^+/valinomycin ratio significantly less than 1 (Table I). When Δ_{max} was calculated from the x-intercept in Fig. 4 using Eqn. 2, the Cs^+/valinomycin ratios

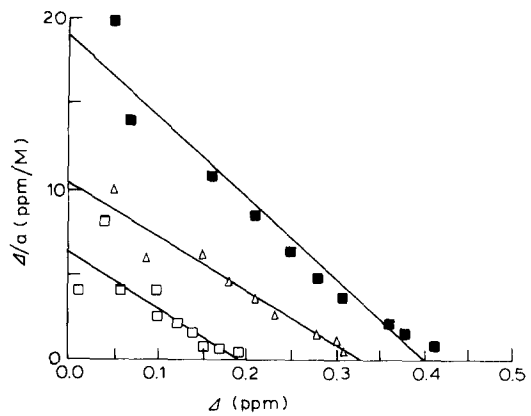

Fig. 3. Plot of Rb^+ binding data, as described in the text, where Δ is the change in chemical shift and a is the activity of Rb^+. The binding constants are given by the negative of the slope of the plots. The maximum shifts are given by the x intercept. The curves represent: □, L-lac α CH; ■, D-hyiv α CH; △, L-val α CH; with binding constants derived from the slopes of 32.9 M^{-1}, 47.8 M^{-1} and 31.7 M^{-1}, respectively. Experiments were performed at 50 °C with 10 mM total DMPC-d_{72} and 3 mol% valinomycin. SCN^- concentration was held constant with NaSCN throughout the titration as described in Materials and Methods.

TABLE I

CATION/VALINOMYCIN RATIOS FROM HILL PLOTS [a]

Valinomycin protons	Rb^+	K^+	Cs^+
Lac α	0.95	1.1	0.73
Hyiv α	0.82	1.1	0.71
L-Val α	0.98	1.1	0.54

[a] Stoichiometries were obtained from the slopes of double log plots of $(\Delta/\Delta_{max})/(1-(\Delta/\Delta_{max}))$ vs. log(cation concentration) to obtain the Hill coefficient, n, from the Hill equation [28]:

$$\frac{\Delta}{\Delta_{max}} = \frac{K[\text{cation concentration}]^n}{1 + K[\text{cation concentration}]^n}$$

In its rearranged form:

$$\frac{\Delta/\Delta_{max}}{1-\Delta/\Delta_{max}} = K[\text{cation concentration}]^n$$

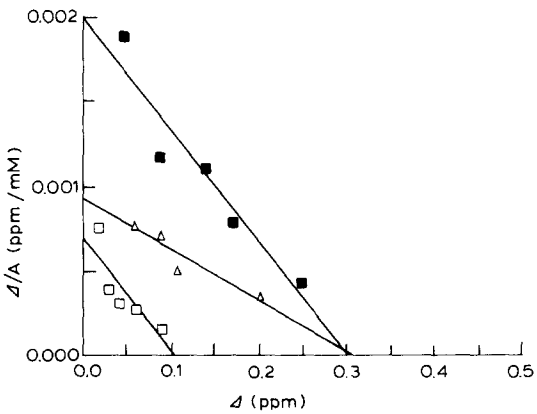

Fig. 4. Plot of Cs^+ binding data, as described in the text, where Δ is the change in chemical shift and a is the activity of Cs^+. The curves represent: □, L-lac α CH; ■, D-hyiv α CH; △, L-val α CH; with binding constants derived from the slopes of 2.02 M^{-1}, 4.25 M^{-1} and 3.33 M^{-1}, respectively. Experiments were performed at 50 °C with 10 mM total DMPC-d_{72} and 3 mol% valinomycin.

were very close to 1. Therefore, the Cs^+ complex must be different from the K^+ and Rb^+ complexes in some respect. Either the maximum shifts in the Cs^+-complexed form are lower than those of the K^+ and Rb^+ forms, or else there is some contribution from Cs^+-valinomycin complexes of stoichiometry other than 1 : 1. Ba^{2+} also exhibited very weak binding to valinomycin, but it was not possible to determine the binding constant from the data or whether the binding was 1 : 1.

The apparent binding constants for the cations can be estimated from the slope of the plots shown in Figs. 2B, 3 and 4. In order to make an order of magnitude estimate of the Cs^+ binding constant, the binding was assumed to be 1 : 1. Values of approx. 37, 22 and 5 M^{-1} were obtained for Rb^+, K^+ and Ca^+, respectively. In Table II, the relative binding constants of various cations for membrane-bound valinomycin, as determined by ^{1}H-NMR, are compared to those measured for 1 : 1 binding in various solvents by ultraviolet spectra, circular dichroism [14,31] or binding of fluorescent dyes [32]. These relative binding constants are defined by setting the binding constant of Rb^+ equal to 1.0 in each different medium and calculating the binding constants of other ions relative to Rb^+. Thus, the cation specificity of valinomycin under various conditions is illustrated by the ratio of a given binding constant to the Rb^+ binding constant under the same conditions.

TABLE II

RELATIVE BINDING CONSTANTS FOR CATION BINDING TO VALINOMYCIN [a]

Cation	Ethanol [b]	Methanol [b]	H_2O [c]	Membrane
Rb^+	1.0	1.0	1.0	1.0
	$(2.9 \cdot 10^6)$ [d]	$(1.8 \cdot 10^5)$	(5.9)	(≈ 2)
K^+	0.89	0.44	0.39	0.59
Cs^+	0.22	0.14	0.22	0.081
Ba^{2+}		0.012		< 0.01
Na^+	$<10^{-5}$	$2.6 \cdot 10^{-5}$		≈ 0
Li^+		$<10^{-5}$		≈ 0

[a] In all cases the relative binding constant was defined as the ratio of a given binding constant to the Rb^+ binding constant under the same conditions.

[b] Data from Ovchinnikov and Ivanov [50].

[c] Data from Feinstein and Felsenfeld [32].

[d] Data in parentheses are the absolute binding constants determined in these systems for Rb^+.

Clearly, the relative order of affinity of membrane-bound valinomycin for these ions is quite similar to that of valinomycin in several solvents.

The binding of SCN^- to the surface of the vesicles imparts a negative surface charge and raises the concentration of cations near the surface [9,33]. Intrinsic binding constants for membrane-bound valinomycin were estimated by extrapolation to zero thiocyanate concentration. The variation in surface charge during the titrations could be eliminated by adding the appropriate amount of NaSCN at the beginning of the experiment in order to hold constant the total SCN^- activity. The effect of SCN^--induced surface charge on the binding of Rb^+ to membrane-bound valinomycin is shown in Fig. 5. It is clear that when RbSCN is titrated into the vesicle preparation in the absence of added NaSCN, the binding is less than at constant SCN^- activity, because of the surface charge effect. In the experiments which allow $[SCN^-]$ to vary, apparent binding constants for Rb^+ can be deduced at any single point of the titration by rearrangement of Eqn. 2 giving:

$$K_{app} = \Delta / ([Rb]_t (\Delta_{max} - \Delta)) \quad (3)$$

This corresponds to drawing a curve in Fig. 5, that

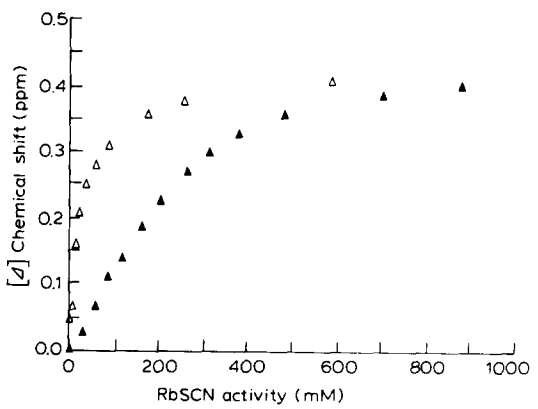

Fig. 5. Binding isotherms of Rb^+ to valinomycin at constant activity of SCN^- (△) and at varying SCN^- activity (▲). The titration of the shift of the D-hyiv α proton was performed in the presence of 2.25 M NaSCN, so that SCN^- concentration remained constant. The varying ionic strength titration began at nearly zero ionic strength and RbSCN was titrated such that the SCN^- activity as well as the Rb^+ activity varied throughout the titration. Experiments were performed at 50 °C with 10 mM total DMPC-d_{72} and 3 mol% valinomycin.

goes through the origin and the appropriate data point with an asymptote at $\Delta = \Delta_{max}$, and that obeys:

$$\Delta = K_{app}[Rb]_t \Delta_{max}/(K_{app}[Rb]_t + 1) \quad (4)$$

In Fig. 6 it can be seen that the apparent binding constants decrease to about 2 M^{-1} near zero SCN^-. This is close to the binding constant of valinomycin for Rb^+ determined in H_2O [32]. Table II shows that the absolute binding constants are quite sensitive to the nature of the solvent in which binding occurs. In view of the similarity of the intrinsic binding constants for valinomycin in H_2O and in the membrane, as well as the sensitivity of binding to surface charge, it is clear that binding of cations by valinomycin is affected by aqueous solvation of the cation and the surface concentration of the cation.

Valinomycin can consequently be used as a ^{1}H-NMR probe to estimate the effective surface concentration of cations. For example, if 2 M^{-1} is the intrinsic binding constant for Rb^+ binding to membrane-bound valinomycin, then the Rb^+ surface concentration can be estimated from:

$$K_{int} = \Delta/([Rb]_{int}(\Delta_{max} - \Delta)) = 2\ M^{-1} \quad (5)$$

$$[Rb]_{int} = \Delta/(K_{int}(\Delta_{max} - \Delta)) = (K_{app}/K_{int})[Rb]_t \quad (6)$$

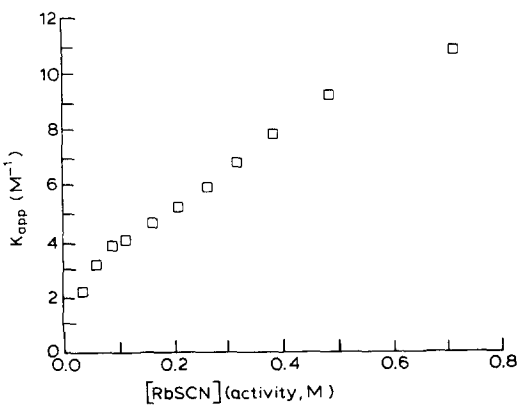

Fig. 6. Dependence of apparent Rb^+ binding constant on SCN^- activity. The apparent interfacial binding constant was determined, as described in the text, from the titration of the D-hyiv α resonance at varying SCN^- activity shown in Fig. 5. By plotting the apparent binding constants against the total SCN^- activity, an index of the SCN^- effect on surface charge and, therefore, apparent binding constant is obtained.

where K_{int} is the intrinsic binding constant, obtained by using the local concentration of the cation near the membrane/aqueous interface, and $[Rb]_{int}$ the interfacial Rb^+ concentration. We note that the only difference between the intrinsic and apparent equilibrium constants, as defined here, is the value used for the cation concentration. The estimated surface concentration of Rb^+ is shown in Fig. 7 as a function of added RbSCN. The surface concentration in the absence of SCN^- would be close to the bulk concentration shown by the lower curve. Clearly, as more SCN^- is introduced into the system, the ratio between the surface concentration and the bulk concentration increases because of greater surface charge.

Location in the membrane

The location and orientation of valinomycin in the membrane were investigated using paramagnetic probes. Pseudocontact (dipolar) interactions with paramagnetic shift reagents change the chemical shifts of nuclei in a strongly distance-dependent and weakly angle-dependent manner [34,35]. Lanthanide cations bound to the membrane surface shift resonances of protons close to the interface more than those deep within the bilayer [36]. This technique has been successfully used to determine the orientation of gramicidin A' in a phospholipid membrane [15,37].

For valinomycin bound to sonicated DMPC

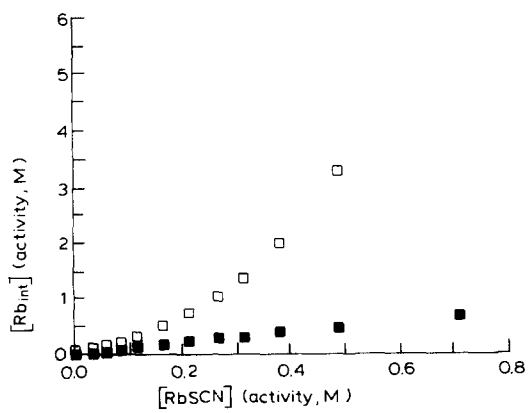

Fig. 7. Effect of SCN^- activity on the surface or interfacial concentration of Rb^+, $[Rb_{int}]$. $[Rb_{int}]$ (□) was determined as described in the text. Data for the plot were those shown in Fig. 5. The bulk concentration of Rb^+ (■) was also plotted in contrast to the surface concentration.

vesicles, the ferricyanide anion was used as a paramagnetic shift reagent in order to avoid the strong interactions possible between cationic shift reagents and valinomycin. Like lanthanides, ferricyanide also binds to the surface of the membrane and shifts resonances of protons near the surface more than those buried in the bilayer interior [38,39]. With shift reagent located uniformly over the surface, we expect an approximate r^{-1} dependence of the pseudocontact shift where r is the distance from the surface to the proton of interest [40]. The data in Fig. 8 show that the induced shifts of the protons near the choline nitrogen are much greater than those of other protons deeper in the membrane. This shift might be due to a contact as well as pseudocontact mechanism. Nonetheless, the induced shifts of valinomycin resonances are similar to those of phospholipid proton resonances in the hydrophobic portion of the bilayer. The slightly downfield shift for these resonances might be due to pseudocontact shift or a bulk susceptibility change caused by the relatively large concentration of ferricyanide (up to 200 mM) used for these experiments [41]. The slightly downfield paramagnetically-induced shift shows that the time average

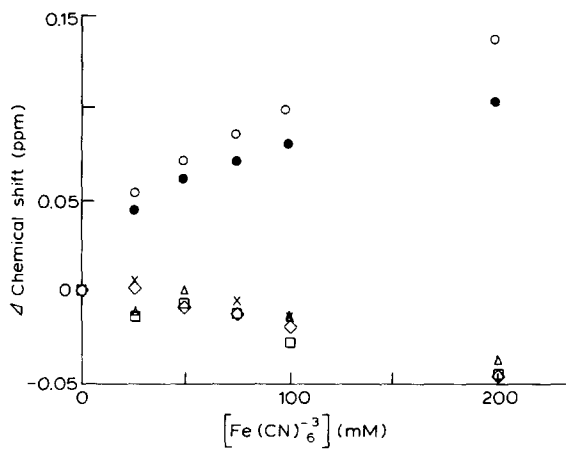

Fig. 8. $Na_3Fe(CN)_6$ titration of the chemical shifts of valinomycin and phospholipid protons: phospholipid headgroup choline methyl (○) and choline methylene (●); the proton on the 2 carbon of the glyceryl (◇); phospholipid acyl chain methyl (+) and methylene (×); valinomycin lactic acid α (□) and hydroxyisovaleric acid α (△). Experiments were performed at 50 °C with 10 mM total DMPC-d_{72} and 3 mol% valinomycin. Chemical shifts in this case were reported with respect to sodium 2,2-dimethyl-2-silapentane 5-sulfonate.

location of uncomplexed valinomycin may be in the hydrocarbon interior of the bilayer.

The distance dependence of the enhancement of T_1 relaxation rate by nitroxide spin labels [42–45] was also used in a simple way to determine the location of valinomycin in the membrane. For a plane of nitroxide spin label at a given depth in the membrane, the effect on protons at another depth has a strong distance dependence of approx. r^{-4} [46]. The proton relaxation times are governed by the relationship

$$(T_1)_{obs}^{-1} = T_1^{-1} + T_{1p}^{-1}$$

where $(T_1)_{obs}^{-1}$ is the observed relaxation rate, T_1^{-1} the unperturbed relaxation rate, and T_{1p}^{-1} the paramagnetic contribution to the relaxation rate from the spin label. Two spin labels were used for these experiments. TEMPO stearate (TS) was chosen for the maximum relaxation enhancement by the unpaired electron in the interfacial region of the membrane. 16-Doxylstearic acid was chosen for the maximum effect near the center of the bilayer [47]. Fig. 9 shows an example of the data obtained from these experiments for two phospholipid resonances. The magnitude of T_{1p}^{-1} rises monotonically with spin label concentration. These data were fitted by straight lines and the slope

TABLE III

PARAMAGNETIC CONTRIBUTION TO RELAXATION BEHAVIOR

Values quoted are paramagnetic contributions to spin label relaxation rate: T_{1p}^{-1}/mol% spin label.

Protons observed	16 label	TS label [a]	16/TS [b] ratio
DMPC CH_3	5.71	3.08	1.85
DMPC CH_2	5.70	4.05	1.41
DMPC αCH_2	7.36	5.65	1.30
DMPC glyc CH	4.43	5.83	0.76
DMPC $N(CH_3)_3$	2.18	5.24	0.42
Val lac α, NaSCN [c]	10.0	7.43	1.35
Val lac α, no salt	7.68	5.72	1.35
Val lac α, KSCN [c]	29.1	29.5	0.99
Val lac α, 343 K	6.32	4.80	1.32

[a] TS, TEMPO stearate.

[b] Ratio of T_{1p}^{-1}/mol% spin label for the 16 label vs. the TS label.

[c] 2.25 M activity.

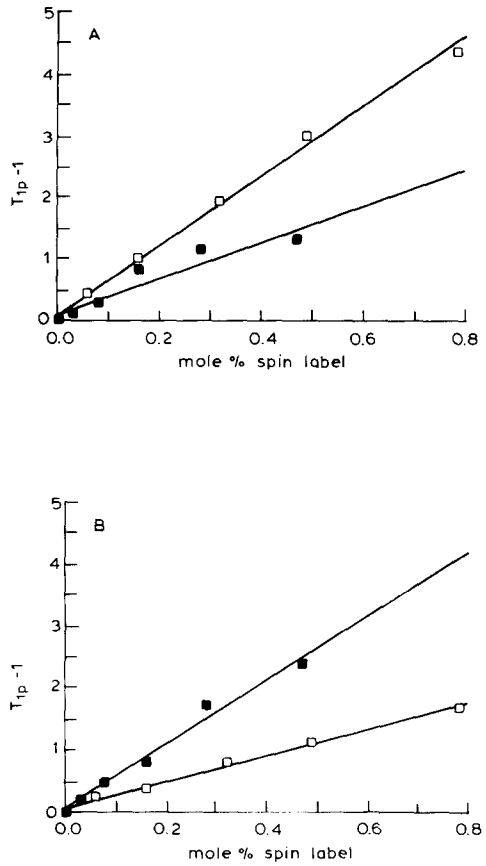

Fig. 9. Effect of 16-doxyl stearate (□) and TEMPO stearate (■) on the relaxation rate (T_{1p}^{-1}) of proton resonances from the terminal methyl proton of the phospholipid fatty acyl chains (A) and from the phospholipid choline methyls (B). Solid lines are linear regression fits to the data. Experiments were performed at 50 °C with 10 mM total DMPC.

used as a measure of the differential effectiveness of relaxation by the spin labels. The absolute value of the slope, T_{1p}^{-1}/mol% spin label for each label was not used for determination of location because of the possible effects of differences in the accessibility or local motions of various protons which would lead to effects on the T_{1p}^{-1} not related to depth within the bilayer. Instead, the ratio of the relaxation rate contributions of the two localized probes, $R_{16/TS}$, was used to estimate depth in the membrane. In Table III, the distance dependence of this relaxation enhancement is shown by the contributions of the labels to the relaxation rates of phospholipid resonances. The effect of the TEMPO stearate label is greatest on resonances from the glycerol backbone and headgroup region, whereas the 16-doxyl stearate label has greatest effect at the terminal methyl and of the acyl chains of the phospholipid. Therefore, the ratio of the paramagnetic contributions to T_1 relaxation rates for the two labels, $R_{16/TS}$, is greater than 1 near the center of the bilayer, and less than 1 near the headgroup.

In order to obtain sufficiently precise data to measure the paramagnetic contribution to T_1 by the inversion-recovery method, it was necessary to use a valinomycin proton resonance which had an unvarying baseline throughout the T_1 experiment. At 323 K, this was the case only for the lac-α proton. For the uncomplexed form of valinomycin in the presence or absence of NaSCN, the value of $R_{16/TS}$ was greater than 1 for the lac-α resonance, and comparable in magnitude to the values seen for the methyl and methylene protons of the acyl chain of the phospholipid (Table III).

At higher temperature, the T_1 of both the L-lac and D-hyiv α protons can be measured reliably because of sharper resonances and because the residual H^2HO resonance shifts upfield, leaving a flatter baseline for the D-hyiv resonance. The effects of the spin labels on the L-lac and D-hyiv α protons are approximately the same ($R_{16/TS} = 1.31$ and 1.25 for lac α and hyiv α, respectively, at 0.25 mol% label). Together with the results of the ferricyanide shift reagent experiments, these experiments are consistent with an average location for the cation-free form of valinomycin deep within the bilayer.

In contrast, upon binding K^+, the value of $R_{16/TS}$ for the valinomycin lac-α proton drops significantly to a value slightly less than 1 (Table III), as is the case for the glycerol backbone region of the membrane. This result indicates that valinomycin changes its average location in the bilayer to an area nearer the headgroup region of the membrane when it binds K^+.

The temperature dependence of T_1 for the α protons in valinomycin in the absence of paramagnetic agents also yields information about the motions governing the relaxation. These α protons can be relaxed most efficiently by vicinal sidechain protons. From the structure of valinomycin it is clear that methyl protons should be largely responsible for the relaxation of the L-lac α pro-

tons. The dependence of T_1 on correlation time of the nearby protons [48] dictates that, if T_1 increases with temperature, the correlation time dominating the relaxation must be shorter than the reciprocal of 2π times the Larmor frequency (approx. $5 \cdot 10^{-10}$ s at 300 MHz). The T_1 values for the L-lac α protons are 0.55, 0.60 and 0.73 s at 300, 323 and 343 K, respectively. Thus, the correlation time dominating relaxation is less than approx. $5 \cdot 10^{-10}$ s. A reasonable candidate for such rapid motion within the bilayer would be axial reorientation of a methyl rotor [49]. A correlation time of approx. 10^{-10} s can be calculated from the equation for T_1 in terms of distance and correlation time for isotropic motion [44] for a stationary α proton relaxed by three protons 0.225 nm away (the distance of closest approach using molecular models) and for an observed T_1 of 0.56 s.

Discussion

Ion-binding

Several facts have emerged from this investigation of cation binding to valinomycin. First, the relative binding affinity of valinomycin for cations is approximately the same in the membrane as in several solvents ($Rb^+ > K^+ > Cs^+ > Ba^{2+}$). Furthermore, the magnitudes of the apparent binding constants for this system are close to those observed in H_2O and are sensitive to surface charge. Also, there is no evidence for cation-valinomycin complexes of stoichiometry other than 1:1 for Rb^+ and K^+.

Only with Cs^+ is there any indication of a possible binding stoichiometry of 1:2, as indicated for the K^+-valinomycin 'sandwich complex' in methanol [10] and the Ca^{2+} complex in acetonitrile [11]. Such a complex also cannot be ruled out for Ba^{2+}. However, the apparent deviation of the Cs^+ data from the 1:1 pattern can also be explained by lower maximum shift changes, as shown for Cs^+ complexes in several solvents [50]. Although we did not detect 1:2 complexes for Rb^+ and K^+ it is possible that such complexes only form at very low cation-to-valinomycin ratios, where they would be difficult to detect because of the weak binding constants. Nonetheless, it is clear that there is no evidence for 1:2 K^+ or Rb^+ complexes with membrane-bound valinomycin when cations are in excess. This could indeed be the situation in negatively charged biological membranes, because a locally high surface concentration of cations would be expected.

The possible use of valinomycin as a probe for the surface concentration of cations is an interesting outcome of these experiments. The experiments presented here do not provide a rigorous test for the accuracy of such a technique. They do, however, provide a possibly useful complementary method to others, such as electrical measurements using black lipid membranes (BLMs) [9].

Location and orientation

The ^{1}H-NMR technique used in these experiments is one of the few ways to obtain information directly about the location and orientation of valinomycin in a membrane. The deep location of the cation-free form of this ionophore suggested by our results differs somewhat from the location deduced by Hsu and Chan [13] from phospholipid ^{1}H-NMR linewidths and relaxation times. The interaction of valinomycin with the hydrocarbon region might not be evident in those indirect experiments in which only phospholipid resonances were observed. On the other hand, the looser packing of chains in sonicated vesicles might allow greater penetration of valinomycin than in the unsonicated, multilamellar vesicles used by Hsu and Chan [13]. The location of valinomycin in the interior of the bilayer is consistent with our earlier ^{1}H-NMR results based on the observed chemical shifts of valinomycin [19], the results of Grell et al. [14] based on circular dichroic spectra, and the conclusions of Finer et al. [12]. In the first two cases, it was shown that the conformation of valinomycin in the bilayer is the same as that in nonpolar solvents.

It is interesting that the binding constants of cations to membrane-bound valinomycin are similar to aqueous binding constants and that binding is sensitive to surface charge, yet free valinomycin resides in the membrane interior. Exchange between cation-free and cation-bound valinomycin is fast on the NMR time scale for these experiments (i.e., faster than approx. 150 s^{-1}) and the cation-free valinomycin molecule spends most of the time in the hydrocarbon region. Rapid excursions to the interface lead to cation binding. The

valinomycin must compete there with water for hydrated cations. This interfacial binding is crucial to valinomycin's activity as an ionophore. There must be an appreciably large off-rate for the cations, or valinomycin would not act as an efficient ionophore because it would hold the cations too long [51]. At the interface the weak aqueous binding constant allows this criterion to be met. The rapid movement of valinomycin between the membrane surface and interior is consistent with the results of Stark et al. [7], who determined that the average time for cation-free or cation-bound valinomycin to cross a black lipid membrane is approx. 10^{-4} s.

To determine the average location of the cation-complexed form of valinomycin, we use the distance dependence of the effect of paramagnetic spin labels on the T_1 relaxation times of valinomycin protons. This analysis utilizes the ratios of the effects of spin labels and leads to the conclusion that valinomycin changes its average location toward the interface upon cation binding. However, it is clear that the absolute magnitudes of the spin label effects are not the same for all protons. For instance, the relaxation enhancements caused by both spin labels are significantly greater for the lac α resonance of the K^+-complexed form of valinomycin (Table II) than for other resonances in the membrane, including the lac α resonance of valinomycin with Na^+ or with no cation bound. Despite this change in valinomycin structure, the ratios of the spin label effects, $R_{16/TS}$, would be relevant indicators of a change in the average location of valinomycin to a depth closer to the aqueous interface (see Addendum).

A change in average location of K^+-bound valinomycin, moving it closer to the headgroup region of the bilayer, is reasonable because valinomycin would be positively charged upon cation binding, assuming that it does not carry a counterion with it. Even with a counterion, the zwitterion complex would be stabilized in a more polar environment. This change in location, or depth, suggests one way in which certain membrane proteins could change conformation on binding cations, i.e., part of the protein bound to the cation could find an energy minimum closer to the interface than its unbound location. This speculation, of course, requires a relatively mobile segment of protein which is normally relatively buried in the bilayer in its free form, yet is accessible to aqueous cations.

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Addendum

There are three major factors which could account for the difference in absolute magnitude of the relaxation enhancements. First, increased exposure of the protons to the spin label would allow a net closer approach and, therefore, increased effect on T_1. Second, a related effect would result from adsorption of the spin labels to the valinomycin molecule. Third, modulation of the spin label magnetic field by a motional frequency closer to the Larmor frequency would affect T_1.

The effect of motion on the modulation of the paramagnetic relaxation contribution is probably dominated by the axial diffusion of the nitroxyl radicals, which is probably close to the proton Larmor precision frequency in these experiments [52,53]. The lateral diffusion of the spin labels in the membrane, which is not close to the Larmor frequency, but is a motion of larger amplitude, probably does not contribute greatly to the relaxation mechanism [54] at this field strength. Since we observed only small differences in the spin label effects on the T_1 values of phospholipid resonances in the presence of Na^+ vs. K^+, it appears that the differing effects on valinomycin in the presence of Na^+ vs. K^+ are probably not related to changes in motion of the spin labels per se as a result of a Na^+ or K^+ effect. Local changes in motion within the valinomycin molecule probably do not affect the paramagnetic relaxation

much because the reorientations of the backbone L-lac and D-hyiv α protons are strongly restricted. They cannot be much faster than the overall molecular tumbling. An estimated rotational correlation time for membrane-bound valinomycin would be longer than 10^{-8} s (for a sphere of radius 10 Å tumbling in a medium of 10–100 centipoise). This is not near the correlation time corresponding to the Larmor frequency (about $5 \cdot 10^{-10}$ s), so that valinomycin motion itself would not modulate efficiently the paramagnetic contribution to the relaxation rate.

It is also unlikely that specific adsorption of the probes to the valinomycin molecule is involved in the difference in T_1 magnitudes observed. If a charge interaction were involved, it would be expected that the free acid, 16-doxylstearic acid would be more likely to adsorb to the positively charged K^+ complex than the ester, TEMPO stearate. Instead, the opposite is observed. Although the magnitude of the effect of both spin labels is greater, it is the TS label which shows the greatest effect on the K^+ complex of valinomycin. Furthermore, since valinomycin (3 mol%) was in excess of the spin label (0–0.3 mol%) in these experiments, one could also rule out the case of strong specific adsorption with slow exchange. This would lead to a small fraction of valinomycin with greatly enhanced relaxation rates and a large fraction with little or no change in relaxation rates. This was not observed.

In view of these facts, it seems likely that an increased exposure of the α protons, allowing closer approach of the spin labels, is the best explanation for the difference in the absolute magnitudes of the paramagnetic spin label contribution to valinomycin T_1 relaxation. It can be seen from the crystal structures [21–23] and molecular models that the lac α protons assume a position further from the center of the valinomycin molecule and apparently more exposed to solvent, as the ester and amide carbonyl carbons bend inward to act as ligands for cations. Furthermore, the access of these protons to the central, less exposed portion of the valinomycin A_2 ring would be hindered by the presence of the cation and the carbonyl oxygens. This increased exposure would explain the large magnitude of the spin label effects on the K^+ form of valinomycin.

References

1 Mueller, P. and Rudin, D.O. (1967) Biochem. Biophys. Res. Commun. 26, 398–404.
2 Ovchinnikov, Yu A. (1974) FEBS Lett. 44, 1–21.
3 Haynes, D.H. and Pressman, B.C. (1974) J. Membr. Biol. 18, 1–21.
4 Eisenman, G., Szabo, G., McLaughlin, S.G.A. and Ciani, S.M. (1972) in Molecular Mechanisms of Antibiotic Action on Protein Synthesis and Membranes (Vasquez, D., Munoz, E. and Garcia-Fernandez, F., eds.), pp. 545–602, Elsevier, Amsterdam.
5 Pressman, B.C., Harris, E.J., Jagger, W.S. and Johnson, J.H. (1967) Proc. Natl. Acad. Sci. USA 58, 1949–1956.
6 Krasne, S., Eisenman, G. and Szabo, G. (1971) Science 174, 412–415.
7 Stark, G., Ketterer, B., Benz, R. and Läuger, P. (1971) Biophys. J. 11, 981–994.
8 Benz, R., Frölich, O. and Läuger, P. (1977) Biochim. Biophys. Acta 464, 465–481.
9 McLaughlin, S., Bruder, A., Chen, S. and Moser, C. (1975) Biochim. Biophys. Acta 394, 304–313.
10 Ivanov, V.T. (1975) Ann. NY Acad. Sci. 264, 221–243.
11 Vishwanath, C.K. and Easwaran, K.R.K. (1982) Biochemistry 21, 2612–2621.
12 Finer, E.G., Hauser, H. and Chapman, D. (1969) Chem. Phys. Lipids 3, 386–392.
13 Hsu, M. and Chan, S.I. (1973) Biochemistry 12, 3872–3876.
14 Grell, E., Funck, Th. and Eggers, F. (1972) in Molecular Mechanisms of Antibiotic Action on Protein Biosynthesis and Membranes, (Munoz, E., Garcia-Fernandez, R. and Vasquez, D., eds.), pp. 646–684, Elsevier, Amsterdam.
15 Feigenson, G.W., Meers, P.R. and Kingsley, P.B. (1977) Biochim. Biophys. Acta 471, 487–491.
16 Kingsley, P.B. and Feigenson, G.W. (1979) FEBS Lett. 97, 175–178.
17 Kingsley, P.B. and Feigenson, G.W. (1979) Chem. Phys. Lipids 24, 135–147.
18 Meers, P.R. and Feigenson, G.W. (1985) J. Lipid Res. 26(7), 882–888.
19 Feigenson, G.W. and Meers, P.R. (1980) Nature 283, 313–314.
20 Bystrov, V.F., Gavrilov, Y.D., Ivanov, V.T. and Ovchinnikov, Y.A. (1977) Eur. J. Biochem. 78, 63–82.
21 Duax, W.L., Hauptman, H., Weeks, C.M. and Norton, D.A. (1972) Science 176, 911–914.
22 Karle, I.L. (1975) J. Am. Chem. Soc. 97, 4379–4386.
23 Smith, G.D., Duax, W.L., Langs, D.A., DeTitta, G.T., Edmonds, J.W., Rohrer, D.C. and Weeks, C.M. (1975) J. Am. Chem. Soc. 97, 7247–7247.
24 Hyman, E.S. (1970) Biophys. J. 10, 72a.
25 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
26 Morrison, W.R. (1964) Anal. Biochem. 7, 218–224.
27 Freier, R.K. (1978) Aqueous Solutions, Vol. 2, pp. 247–251, Walter de Gruyter, New York.
28 Vold, R.L., Waugh, J.S., Klein, M.P. and Phelps, D.E. (1968) J. Chem. Phys. 48, 3831–3832.
29 Hauser, H. (1976) J. Coll. Int. Sci. 55, 85–93.

30 Hill, A.V.J. (1910) J. Physiol. (London) 40, iv–vii.
31 Shemyakin, M.M., Ovchinnikov, Yu.A., Ivanov, V.T., Antonov, V.K., Vinogradova, E.I., Shkrob, A.M., Malenkov, G.H., Evstratov, A.V., Laine, I.A., Melnik, E.I. and Ryabova, I.D. (1969) J. Membr. Biol. 1, 402–430.
32 Feinstein, M.B. and Felsenfeld, H. (1971) Proc. Natl. Acad. Sci. USA 68, 2037–2041.
33 Jendrasiak, G. (1972) Chem. Phys. Lipids 9, 133–146.
34 Hinckley, C.C. (1969) J. Am. Chem. Soc. 91, 5160–5162.
35 Bergel'son, L.D. (1976) Z. Vses. Khim. Ob-va im. Mendeleeva 21, 738–750.
36 Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1976) Nature 261, 390–394.
37 Weinstein, S., Wallace, B.A., Blout, E.R., Morrow, J.S. and Veatch, W. (1979) Proc. Natl. Acad. Sci. USA 76, 4230–4234.
38 Kostelnik, H.J. and Castellano, S.M. (1972) J. Magn. Res. 7, 219–223.
39 Berden, J.A., Barker, R.W. and Radda, G.K. (1975) Biochim. Biophys. Acta 375, 186–208.
40 Meers, P. (1983) Ph.D. Thesis, Cornell University.
41 Live, D.H. and Chan, S.I. (1970) Anal. Chem. 42, 791–792.
42 Solomon, I. (1955) Phys. Rev. 99, 559–565.
43 Bloembergen, N. (1957) J. Chem. Phys. 27, 572–573.
44 Abragam, A. (1961) The Principles of Nuclear Magnetism, Chapter 8, Oxford University Press, Oxford.
45 Kornberg, R.D. and McConnell, H.M. (1971) Proc. Natl. Acad. Sci. USA 68, 2564–2568.
46 Brown, L.R., Bösch, C. and Wüthrich, K. (1981) Biochim. Biophys. Acta 642, 296–312.
47 Godici, P.E. and Landsberger, F.R. (1974) Biochemistry 13, 362–368.
48 Bloembergen, N. (1961) Nuclear Magnetic Relaxation, Chapter 4, W.A. Benjamin, New York.
49 Feigenson, G.W. and Chan, S.I. (1974) J. Am. Chem. Soc. 96, 1312–1319.
50 Ovchinnikov, Yu A. and Ivanov, V.T. (1976) Z. Vses. Khim. Ob-va im. Mendeleeva 21, 615–625.
51 Lehn, J.M. (1973) Struct. Bond. 16, 1–69.
52 Hubbell, W.L. and McConnell, H.M. (1969) Proc. Natl. Acad. Sci. USA 63, 16–22.
53 Hubbell, W.L. and McConnell, H.M. (1969) Proc. Natl. Acad. Sci. USA 64, 20–27.
54 Brulet, P. and McConnell, H.M. (1975) Proc. Natl. Acad. Sci. USA 72, 1451–1455.