Nuclear Magnetic Resonance Studies of the Interaction of Valinomycin with Unsonicated Lecithin Bilayers†

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ABSTRACT: The interaction of valinomycin with *unsonicated* lecithin bilayers has been investigated by delayed Fourier transform proton magnetic resonance and pulsed nuclear magnetic resonance spectroscopy. The results indicate that valinomycin interacts with dipalmitoyllecithin bilayers predominantly in the region of the polar head groups. No evidence was obtained for gross disruption of the hydrophobic region of the phospholipid bilayer in this case. With dimyristoyllecithin, the nmr data suggest penetration of valinomycin molecules into the hydrophobic core of the bilayer. The difference in behavior between the two lecithins is attributed to the known variation of the bilayer stability with fatty acid chain length. Previous findings on the interaction of valinomycin with *sonicated* phospholipid bilayers (Finer *et al.*, *Chem. Phys. Lipids 3*, 386 (1969)) are discussed in terms of the effects of sonication on the internal structure of lecithin bilayers.

During the past few years, various efforts have been made to understand the functional mechanism of ion transporting antibiotics in membranes. Valinomycin, a cyclodepsipeptide antibiotic, is known to increase alkali ion permeability of mitochondria (Pressman, 1968) and phospholipid liposomes (Johnson and Bangham, 1969), and to increase the conductivity of black lipid films (Stark and Benz, 1971; Stark *et al.*, 1971; Krasne *et al.*, 1971). Solution studies have shown that valinomycin complexes alkali ions (Ivanov *et al.*, 1969; Haynes *et al.*, 1969, 1971) and X-ray diffraction studies (Pinkerton *et al.*, 1969; Duax *et al.*, 1972) of the K^+–valinomycin complex have revealed that the ion is coordinated to the six centrally directed carbonyl groups of the cyclodepsipeptide molecule. Although it is generally believed that valinomycin acts as a carrier for the potassium ion in the antibiotic-induced ion transport across the membrane, little is known about the mechanistic details of this process.

This paper concerns a nuclear magnetic resonance (nmr) study of the interaction of valinomycin with unsonicated phospholipid bilayers. A similar study on sonicated phospholipid bilayers has been reported by Finer *et al.* (1969). However, in view of the recent work of Sheetz and Chan (1972), which demonstrated the effect of vesicle curvature on bilayer structure, a comparative study of the interaction of cyclic antibiotics with unsonicated bilayers and with sonicated vesicles would seem to be in order. In the past, the broad resonances observed for the multilamellar structure have hampered studies with unsonicated bilayers. But this problem has recently been circumvented by the use of delayed Fourier transform (DFT) nmr spectroscopy (Chan *et al.*, 1971; Seiter *et al.*, 1972). The conventional continuous wave proton magnetic resonance (pmr) spectrum of unsonicated phospholipid bilayers consists of two relatively sharp resonances due to the choline methyl and chain terminal methyl protons superimposed on a broad resonance (~3000 Hz) arising from the fatty acid chain methylene protons (Chan *et al.*, 1971; Seiter *et al.*, 1972). With DFT pmr, a receiver dead time is introduced between the end of the radiofrequency pulse and the start of data collection. Accordingly, the broad resonance can be filtered out from the resonance spectrum, if the receiver dead time exceeds the spin–spin relaxation time (T_2) associated with these protons. This technique isolates the sharper resonances and permits reliable line width and intensity measurements on them. Recent reports (Chan *et al.*, 1971; Seiter *et al.*, 1972) exploiting this method have permitted the characterization of the mobility of choline methyl and chain terminal methyl groups of lecithin bilayers in the unsonicated state. It should be noted that the broad methylene resonance can be studied independently by conventional pulsed nmr spectroscopy (Chan *et al.*, 1971), and in the present work on the interaction of the valinomycin with unsonicated lecithin bilayers, we have monitored the methylene protons as well.

Two phosphatidylcholines of different fatty acid chain length have been used in our experiments, dipalmitoyllecithin and dimyristoyllecithin. Other factors being equal, the stability of phospholipid bilayers is dependent upon fatty acid chain length (Chapman *et al.*, 1967). For this reason, we felt that a comparative study of the interaction of valinomycin with dipalmitoyl- and dimyristoyllecithin might reveal the nature of those forces which are important in the interaction of this cyclodepsipeptide with phospholipid bilayers.

Experimental Section

L-α-Dipalmitoyl- and L-α-dimyristoyllecithin were purchased from General Biochemicals and Nutritional Biochemicals Co., respectively. Both were purified with silicic acid column chromatography (Robles and Van den Berg, 1969); 100 g of silicic acid was used per gram of lecithin. A 2.5 × 50 cm column was used and it was eluted with the following solutions: 400 ml of $CHCl_3$; 400 ml of $CHCl_3$–CH_3OH (9:1); and 1000 ml of $CHCl_3$–CH_3OH (1:9). Lecithins were found in the last 1000-ml solution. After this portion of solution was evaporated to dryness, the purity of the lecithins was determined by thin-layer chromatography ($CHCl_3$–CH_3OH–H_2O (65:25:4)). Reagent grade methanol and chloroform were distilled prior to use in the lecithin purification.

The above purified lecithin was dissolved in chloroform

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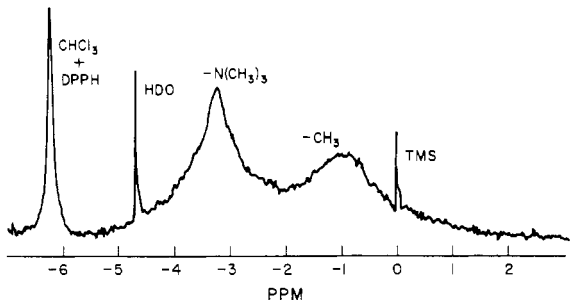

FIGURE 1: Delayed Fourier transform pmr spectrum of unsonicated dipalmitoyllecithin bilayer at 50° and 100 MHz. The receiver deadtime used in obtaining this spectrum is 384 μsec. The chloroform resonance is used as an intensity standard.

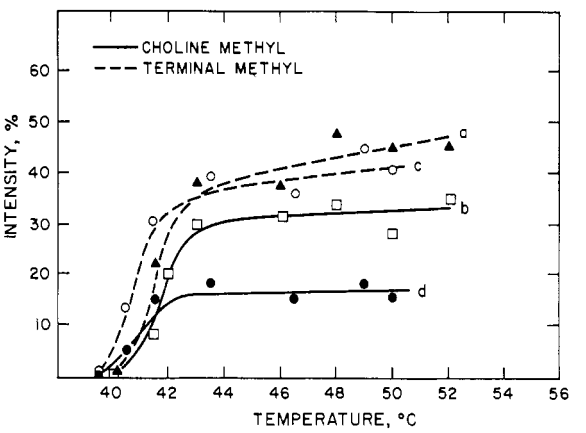

FIGURE 2: Variation of the choline and terminal methyl signal intensities with temperature for unsonicated dipalmitoyllecithin bilayer samples (40% w/v). Curves a and b are for a sample without valinomycin. Curves c and d are for a sample with 2 mol % valinomycin.

in a 5-mm nmr tube. It was then pumped to dryness for a period of 48 hr. In order to get rid of the last trace of methanol used in the chromatography purification, it was found necessary to heat the sample to 50–60°. After transferring in 100% D_2O under vacuum by evaporation–condensation, the tube was sealed and the lecithin suspension was shaken along the length of the tube with a Vortex mixer to produce a homogeneous suspension at temperatures above the crystal $\rightleftharpoons$ liquid crystal phase transition of the lecithin. For lecithin–valinomycin suspensions, the appropriate amount of valinomycin was mixed with the lecithin in chloroform. The mixture was then pumped to dryness as above. Unless otherwise specified, the suspension is 40% (w/v) in lecithin.

The DFT spectra were recorded with a Varian HA-100 nmr spectrometer equipped with Fourier transform accessory and interfaced to a Varian 620i computer. The intensities of the resonance signals were measured against a calibrated chloroform capillary doped with the free-radical 2,2-diphenyl-1-picrylhydrazyl. All intensities are corrected for the receiver dead time introduced into the spectrometer. The sample temperature was controlled by a variable-temperature control unit and was determined from the spectrum of either methanol or ethylene glycol contained in a small sealed capillary immersed inside the nmr tube. Spin–spin relaxation times (T_2) were measured at 13 kG using a pulsed attachment purchased from Tomlinson Research Instrument Co.

Results and Discussion

The DFT pmr spectrum of a 40% dipalmitoyllecithin suspension in D_2O taken with a receiver dead time of 384 μsec is shown in Figure 1. Since the methylene protons have an effective T_2 of ~120 μsec (Chan *et al.*, 1971), only 3% of the methylene proton signal should appear in the DFT spectra for a data collecting delay time of 384 μsec. Thus, the methylene signal is essentially filtered out in the DFT spectrum. The peaks centered at −1.0 and −3.2 ppm in the DFT spectrum have been assigned to terminal methyl protons and choline methyl protons, respectively (Seiter *et al.*, 1972).

The crystalline $\rightleftharpoons$ liquid crystalline phase transition of the dipalmitoyllecithin bilayer was followed by monitoring the intensities of the two methyl resonances as a function of temperature. The temperature of this phase transition (Figure 2), 41.5°, as monitored by this method, is in agreement with that determined by differential thermal analysis (Phillips *et al.*, 1970). The DFT spectrum revealed no sharp components for the crystalline phase, but both the choline and terminal methyl groups were found to be simultaneously mobilized near the bilayer phase transition temperature. However, no more than 50% of the total intensity was observed for both the choline methyl and terminal methyl protons (Figure 2).

It is important to comment on the intensities of the choline methyl and terminal methyl proton resonances observed in the DFT pmr spectrum of the unsonicated lecithin bilayers above the bilayer phase transition. Spin–lattice relaxation measurements of these methyl protons have shown (G. W. Feigenson, C. H. A. Seiter, and S. I. Chan, 1972, unpublished data) that these methyl groups do undergo rapid orientation about their respective top axis under these conditions. However, such an assembly of methyl tops should give rise to a powder pmr spectrum with a sharp central resonance superimposed on a broad background. As shown by Andrew and Bersohn (1950), the sharp spike corresponds to 50% of the total intensity and should exhibit no inhomogeneous broadening in the absence of dipolar interactions with other nuclei, and other broadening mechanisms such as those arising from chemical shift anisotropy and variations in the diamagnetic susceptibility across the sample. In the case of lecithin bilayers, the major contribution to the line width of the central component of the methyl spectrum should come from the dipolar coupling with the neighboring methylene protons on the same fatty acid chain, and thus we expect this line width to depend on the extent to which this interaction is modulated spatially as well as in time. We note that any motion of the fatty acid chain which renders reorientation of the methyl rotor *axis* over a large range of solid angles with respect to the external magnetic field has the effect of gradually collapsing the broad resonance into the central "spike." The extent to which this occurs depends, of course, on the degree of motional restriction and to a lesser extent on the time scale of this motion. In the limit where the reorientation of the rotor axis is unhindered and the motion is sufficiently rapid, for example, the central resonance would account for the total methyl intensity. In view of this background information, we can therefore conclude that the motion of the choline methyl and terminal methyl groups in unsonicated lecithin bilayers is restricted even above the bilayer phase transition. A detailed theoretical analysis of the line shape and intensity of a methyl top which undergoes restricted and anisotropic motion has been worked out in this laboratory and will be published elsewhere (Seiter and Chan, 1973).

As shown in Figure 2, the effects of 2 mol % valinomycin

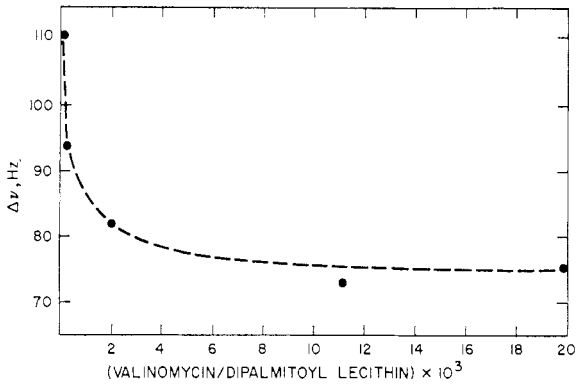

FIGURE 3: Variation of the line width of the choline methyl proton signal with the concentration of valinomycin in an unsonicated dipalmitoyllecithin bilayer sample at 50°.

on the bilayer phase transition of dipalmitoyllecithin are to broaden the phase transition somewhat and to lower the temperature of the phase transition by ~1°. A change in the intensity of the choline methyl resonance was also noted above the phase transition. At the valinomycin concentration of 2 mol % (1 valinomycin/50 molecules of lecithin), only ~20% of the choline methyl protons was accounted for in the choline methyl signal. This intensity drop was accompanied by a concomitant change in the line width. The line width of the choline methyl resonance is presented as a function of the valinomycin concentration in Figure 3. The effect of added valinomycin on the choline resonance can be seen to be very abrupt, with the line width decreasing from 110 to 85 Hz over the narrow concentration of 0–0.1 mol % valinomycin and leveling off at a limiting line width of ~75 Hz. By contrast, there is no detectable effect of the added valinomycin on the line width of the terminal methyl resonance, and no intensity loss was observed for the terminal methyl resonance.

A comparison of the free induction decay of a dipalmitoyllecithin sample in the absence and presence of valinomycin is given in Figure 4. These data reveal no observable effect of added valinomycin on the spin–spin relaxation of the methylene protons of the fatty acid chains, which account for 70% of the protons in the sample.

The above observations indicate that valinomycin interacts with the bilayer predominantly in the region of the polar head groups. The line-width data summarized in Figure 3 are reminiscent of an absorption isotherm, and we believe the observed variation of the line width with valinomycin concentration reflects the partitioning equilibrium of the cyclodepsipeptide between the aqueous and bilayer phases. In all probability, however, the concentration of the antibiotic "dissolved" in the hydrocarbon core of the bilayer is not high, since we obtained no evidence for any gross perturbation in the molecular packing of the hydrocarbon chains. Thus, it seems that either the interacting valinomycin molecules are merely physically adsorbed on or near the surface of the bilayer, or that whatever disruption of the bilayer structure as a consequence of any penetration of the cyclodepsipeptide is confined to the polar head group region at the bilayer–water interface.

Two factors must be considered to account for the line-width decrease and intensity drop of the choline methyl proton signal brought about by the interaction of valinomycin: the effect of chemical exchange and the effect of valinomycin on the mobility of the polar head group. The intensity

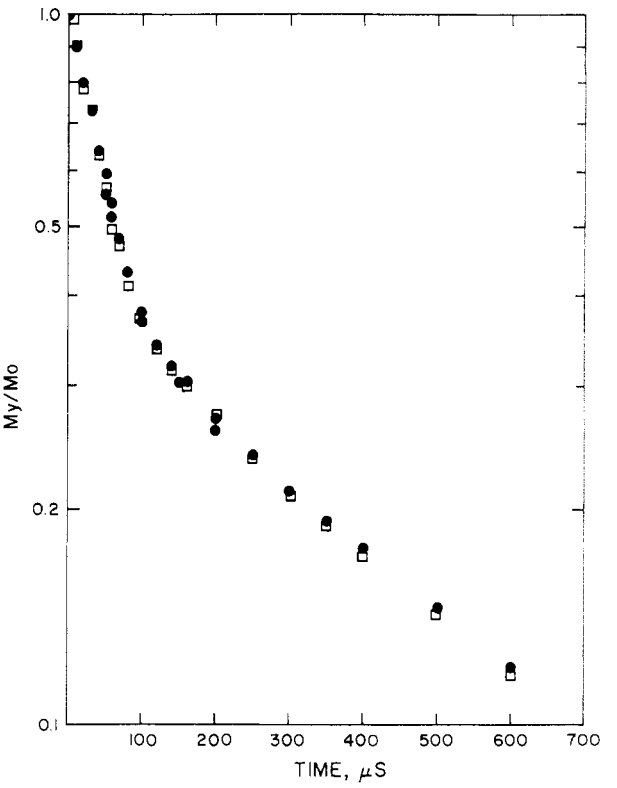

FIGURE 4: The proton free induction decay of unsonicated dipalmitoyllecithin bilayer samples at 50° in a magnetic field of 13 kG: (●) sample without valinomycin; (□) sample with 2 mol % valinomycin.

drop of the choline methyl resonance could only be accounted for by a slow equilibrium between bound and free sites. On the other hand, the narrower line width of the choline methyl signal indicates that the segmental motion of at least some of the polar head groups have become less restricted and possibly more isotropic. Thus, the valinomycin binding has the effect of immobilizing some of the choline head groups while enhancing the mobility of the others. On the basis of these observations, it is not possible to ascertain whether a bound valinomycin molecule mobilizes or immobilizes the polar head groups of phospholipid molecules in its immediate vicinity. Since the effect of the cyclodepsipeptide on the line width and intensity of the choline head groups becomes saturated at a valinomycin concentration of 1 valinomycin/1000 lecithin molecules, the binding of a valinomycin molecule apparently produces a long range effect which is propagated over a distance ten times the diameter of the cyclodepsipeptide ring, if one can assume that the antibiotic is uniformly distributed over the surface of the bilayer.[1] This conclusion is all the more amazing when one considers that we have obtained no evidence for gross disruption of the hydrophobic region of the phospholipid bilayer. This result, we feel, reflects the cooperative nature of the polar head group interactions in a lecithin bilayer.

Somewhat similar observations have been noted above the crystalline $\rightleftharpoons$ liquid crystalline transition for dimyris-

[1] In deducing this result, the molecular area of a lecithin molecule on the surface of a bilayer has been taken to be ~50 Å² (Chapman *et al.*, 1967), and the cyclodepsipeptide ring is assumed to have a cross section area of ~200 Å² (Shemyakin *et al.*, 1969).

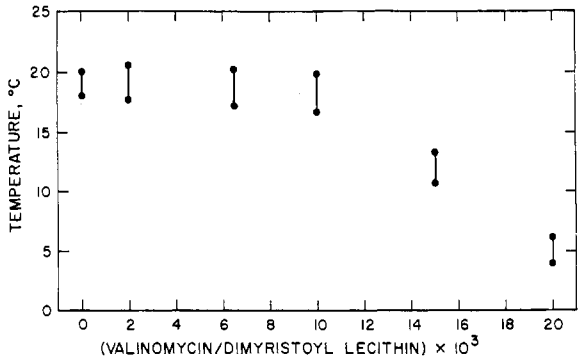

FIGURE 5: Variation of the onset of the crystalline ⇌ liquid crystalline transition temperature with the concentration of valinomycin in unsonicated dimyristoyllecithin bilayer samples.

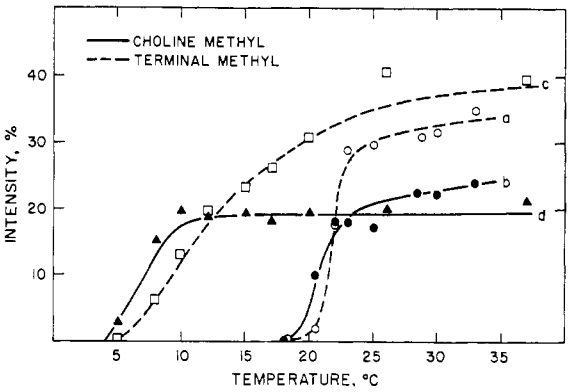

FIGURE 6: Variation of the choline and terminal methyl signal intensities with temperature for unsonicated dimyristoyllecithin samples. Curves a and b are for a sample without valinomycin. Curves c and d are for a sample with 2 mol % valinomycin.

toyllecithin at valinomycin concentrations lower than 1 mol %, except that the observed line width and intensity decrease of the choline methyl signal are smaller than that observed in the case of dipalmitoyllecithin under the same conditions. However, as the valinomycin concentration was increased above 1 mol %, we noted that the crystalline ⇌ liquid crystalline phase transition became shifted to lower temperatures. The extent to which this occurred depended on the valinomycin concentration. The effect of valinomycin on the onset of the phase transition is shown in Figure 5. The transition is also observed to be broadened, and to occur over a broader range of temperatures for the chain methyls than for the choline head groups (Figure 6). We attribute these observations to the differential effect of the added valinomycin on the activities of the lecithin molecules in the crystalline and liquid crystalline phases. The depression in the phase transition temperature indicates that valinomycin is being incorporated into the liquid crystalline phase to a greater extent than in the crystalline phase. The observed variation of the onset of the phase transition temperature with valinomycin concentration clearly reflects the amount of valinomycin which is incorporated into the bilayer at the various concentrations of the antibiotic employed. Significant incorporation of valinomycin molecules within the hydrophobic regions of the bilayer structure was not observed in the case of dipalmitoyllecithin, even at valinomycin concentrations of ~2 mol %, and we surmise that this difference in behavior between the two lecithins reflects the lower stability of the bilayer structure in the case of dimyristoyllecithin. As Phillips *et al.* (1969) have previously noted, the shorter hydrocarbon chains in dimyristoyllecithin render the bilayer structure less stable in this case. From calorimetric measurements, these workers reported enthalpy changes of 8.66 and 6.64 kcal/mol for the crystalline ⇌ liquid crystalline transition in dipalmitoyl- and dimyristoyllecithin, respectively.[2] In this connection, it is noteworthy that we have also observed ~10% *increase* in the transverse relaxation time T_2 for the protons of the polymethylene chains upon the addition of 2 mol % valinomycin to dimyristoyllecithin above the phase transition temperature. This datum could be cited as evidence for penetration of the cyclodepsipeptide into the hydrocarbon regions of the lecithin bilayer; however, it should be pointed out that this T_2 increase is just barely outside the experimental error of our measurements. With the possibility of incorporation of valinomycin molecules within the hydrocarbon region of the bilayer, it is conceivable that deeper penetration of the antibiotic molecules can accompany any additional loosening up of the bilayer structure as the temperature is increased, and this in turn could account for the more gradual "melting" of the hydrocarbon chains indicated by the temperature behavior of the chain methyl group resonance. Also, we suspect that any cooperative effect associated with the binding of a valinomycin molecule to a lecithin bilayer, such as the lateral propagation of structural perturbation induced when a valinomycin molecule is bound to dipalmitoyllecithin bilayers, is dictated by the rigidity of the bilayer structure. In view of the decreased lateral rigidity of the bilayer in the case of dimyristoyllecithin, any structural perturbation of the bilayer as a result of valinomycin binding is not expected to propagate over the large distances inferred in the case of dipalmitoyllecithin, and accordingly, we expect smaller line width and intensity changes for the choline methyl signal in the case of dimyristoyllecithin, as observed.

A similar nmr study on the interaction of valinomycin with sonicated phospholipid has been reported by Finer *et al.* (1969). In this work they observed broadening of both the choline methyl and the fatty acid chain proton signals, indicating that the antibiotic is being incorporated into the hydrophobic core of the sonicated vesicles. This result is perhaps understandable in view of the recent work by Sheetz and Chan (1972), which showed that the packing of the lecithin molecules within a sonicated vesicle is significantly looser than that in the case of unsonicated multilayers. It might be that this looser structure allows the antibiotic to further penetrate into the bilayer. In our judgment, the present conclusions are probably more relevant to the situations in biomembranes, since the vesicle curvature of biomembranes should, in general, correspond more closely to that of unsonicated bilayers than 300-Å vesicles.

This study indicates that valinomycin is a surface active molecule, as has been previously suggested by Shemyakin

[2] We argue that the ΔH of the phase transition provides a more meaningful indication of the stability of the bilayer than the phase transition temperature, as the latter depends on both ΔH and ΔS of the transition. Since both ΔH and ΔS depend on the chain length and the degree of unsaturation of the hydrocarbon chains (Phillips *et al.*, 1969), the phase transition temperature varies with the nature of the lecithin in a subtle way and provides a meaningful indication of the stability of the bilayer only when either the chain length or the degree of unsaturation of the hydrocarbon chains is held fixed.

et al. (1969), and that it interacts with a phospholipid bilayer primarily by adsorption near the bilayer–water interface. Although our data clearly indicate that there is some interaction of this cyclodepsipeptide with the choline head groups, this interaction is apparently not sufficient to affect the state of the hydrophobic core. There is no evidence to suggest that this interaction of valinomycin with the bilayer is altered in the presence of K^+, as the addition of K^+ to a valinomycin–lecithin dispersion was found to have no effect on the DFT pmr spectrum of the lecithin (Seiter *et al.*, 1972). In addition, Stark *et al.* (1971) have shown that the rate of valinomycin transport across a black lipid film is independent of the state of its complexation with K^+. These conclusions are seemingly paradoxical to the carrier mechanism of ion transport for this antibiotic. However, in order to account for the conductivity enhancement of a black lipid film by valinomycin, the concentration of this cyclodepsipeptide inside the bilayer region need not be higher than 10^{-7} M (Stark and Benz, 1971; Stark *et al.*, 1971). Such a low concentration of valinomycin inside the bilayer is, of course, not expected to disrupt the bilayer in a major way. On the other hand, a high concentration of valinomycin on or near the bilayer surface seems necessary to ensure that the antibiotic-mediated ion transport is not limited by the kinetics of ion binding to the cyclodepsipeptide. In order to account for the observed cation selectivity of the valinomycin induced ion permeability, it is usually assumed that a rapid equilibrium exists between the alkali ion in the bulk aqueous phase and the ionophore complex at the bilayer–water interface (Eisenman, 1968), at least on a time scale comparable to the rate of the translocation of the complex across the hydrocarbon barrier. The adsorption of valinomycin molecules near the water–bilayer interface would satisfy this requirement. Thus, valinomycin seems to have a bifunctional role in the antibiotic-mediated ion transport. First, it serves as an ion carrier by virtue of its strong affinity for alkali ions and its solubility in a hydrocarbon-like medium. Secondly, it is a surface active molecule which, *via* ion complexation, renders the bilayer surface more favorable to an excess concentration of alkali ions.

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