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Interaction of Carrier Ionophores with Phospholipid Vesicles†

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ABSTRACT: The interactions of carrier ionophores, nonactin, A23187, and lasalocid A with liposomes formed from the synthetic lipids dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine are investigated by differential scanning calorimetry and ^1H and ^{31}P nuclear magnetic resonance techniques. The results indicate that the mode of interaction of these ionophores is dependent on the fluidity of the bilayer and on the chemical nature of these ionophores. The ^{31}P NMR studies are suggestive of the formation of small particles that are probably intervesicular lipid–ionophore aggregates in multilamellar vesicles when they are incorporated with these ionophores at high concentrations. The results are interpreted on the basis of the chemical structure and conformations of the ionophores in membrane mimetic media. The ^1H NMR line-width measurements indicate that the aromatic rings containing the carboxyl groups of lasalocid A and A23187 are located near the membrane interface while the rest of the molecule is buried in the membrane interior.

A prerequisite for understanding of the ionophore-mediated cation transport across membranes is a detailed knowledge of the preferred conformations of the ionophores, their cation complexes in solution, and their interaction with model and biological membranes. During the last few years, several studies have been aimed at understanding the structure and conformations of ionophores and their cation complexes, and these results clearly demonstrated that the cation selectivity of the ionophores depends not only on the nature of ligands they contain but also on the conformation of the ionophore–cation complex as an entity and this, in turn, is dependent on size and charge of the cation, the counterion (i.e., anion), and the solvent environment (Alpha & Brady, 1973; Chen & Springer, 1978; Degani et al., 1973; Degani & Friedman, 1974, 1975; Devarajan et al., 1980; Easwaran, 1985; Hamilton et al., 1981; Haynes & Pressman, 1974; Johnson et al., 1970; Ovchinnikov et al., 1974; Sankaram & Easwaran, 1982, 1985; Shastri & Easwaran, 1984; Shastri et al., 1987; Vishwanath & Easwaran, 1982, 1983, 1985; Young & Gomperts, 1977). However, studies dealing with the interaction of these ionophores with model and biological membranes are limited though some studies on the interaction of valinomycin (VM)[1] with model membranes have been reported (Hsu & Chan, 1973; Grell et al., 1974; Walz, 1977, 1979; Feigenson & Meers, 1980; Sankaram & Easwaran, 1984). In this paper, we report our results on the interaction of nonactin (NA), lasalocid A (LS), and A23187 (see Figure 1 for chemical structures of the ionophores) with liposomes (both unilamellar and multilamellar vesicles) made from two synthetic lipids, namely, dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) studied with differential scanning calorimetry (DSC) and ^1H and ^{31}P nuclear magentic

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[1] Abbreviations: CD, circular dichroism; CSA, chemical shift anisotropy; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; LS, lasalocid A; MLVs, multilamellar vesicles; NA, nonactin; NMR, nuclear magnetic resonance; ULVs, unilamellar vesicles; VM, valinomycin.

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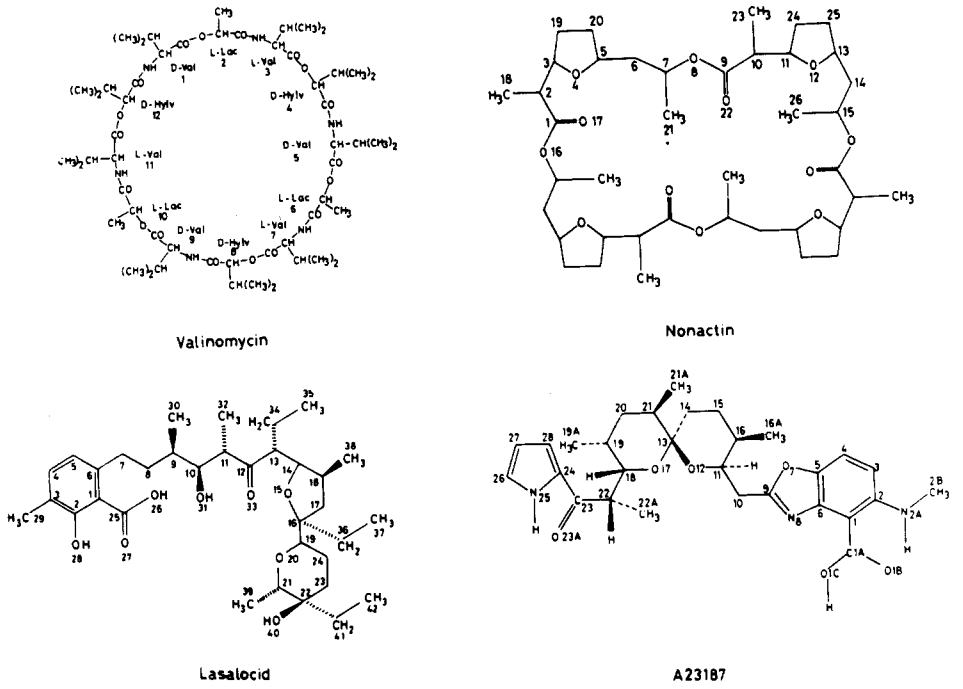

FIGURE 1: Chemical structures of the carrier ionophores.

resonance (NMR) techniques.

The basic strategy of the experiments involves the use of multilamellar vesicles (MLVs) preincorporated with known concentrations of the ionophores for DSC and ^{31}P NMR studies and unilamellar vesicles (ULVs) for ^{1}H NMR linewidth measurements. The lipid bilayer phase transition temperatures and their enthalpies of ionophore-incorporated MLVs were measured by DSC to obtain information on the effect of ionophores on lipid bilayer packing, orientations of the lipid long-chain axes with respect to the bilayer normal, and intravesicular interactions. The effect of ionophores on the ^{31}P NMR chemical shift anisotropy (CSA) parameter ($\Delta\sigma$) was studied to obtain information on the orientation and dynamic changes of the head groups and the morphology of aggregate structures. Further, ^{1}H NMR line widths of ULVs incorporated with the ionophores have been mesured, and the ionophore-induced changes are manifested in the head group choline proton signals and/or the interior methylene proton signals.

Materials and Methods

Sodium salt of lasalocid A, valinomycin, nonactin, A23187, L-α-dimyristoylphosphatidylcholine, and L-α-dipalmitoylphosphatidylcholine were obtained from Sigma Chemical Co. and used without further purification. Deuteriated solvents methanol-d_4 and D_2O were obtained from Stohler Isotopes. Free acid of LS was prepared from its sodium salt as reported in the literature (Alpha & Brady, 1973) and recrystallized from 99% ethanol. Deionized double-distilled water was used for DSC experiments.

^{1}H NMR spectra of the lipid samples for the studies on the location of the ionophores were recorded on a Varian FT-80A NMR spectrometer equipped with a variable-temperature accessory. The proton-decoupled ^{31}P NMR spectra were recorded on a Bruker WM-500 NMR spectrometer operating at a frequency of 187.5 MHz, and the ^{31}P signals of lipid samples were referenced with respect to an external standard of 85% H_3PO_4. In all these experiments, the field frequency lock was provided by the solvent D_2O.

DSC experiments on lipid samples were performed on a Perkin-Elmer DSC-2C instrument with samples sealed in aluminum pans used for volatile samples. The instrument was calibrated with standard samples (Indium and benzene) covering the entire range of temperature. The thermograms were recorded at a heating rate of 2.5 deg/min and sensitivity range of 1 mcal/s. For the calculation of enthalpy values, the thermograms were traced on to a paper of uniform thickness, cut, and weighed.

MLVs used for ^{31}P NMR and DSC studies were prepared as reported by Bangham et al. (1974). A known amount of lipid was dissolved in chloroform, and the solvent was completely removed by flushing with nitrogen gas to form a thin layer in a round-bottom flask and dried overnight under vacuum. The lipid film thus obtained was dispersed in the aqueous medium and shaken thoroughly on a vortex mixer during which the sample temperature was maintained 10 deg above the gel to liquid crystalline phase transition temperature of the lipid. Ionophores were added as chloroform solutions prior to evaporation of the solvent. Typical concentrations of the lipid used were 40 and 60 mM for DSC and ^{31}P NMR studies, respectively.

ULVs for ^{1}H NMR studies were obtained by ultrasonic irradiation of the MLVs prepared as above (Szoka & Papahodjopoulos, 1980, 1981). MLVs were prepared in D_2O and sonicated on a Branson B-12 model sonicator with a microtip at a power level of 60% for 10 min with 10-s pulses, maintaining the sample temperature about 10 deg above the chain melting transition temperature of the lipid. Ionophores were added as methanol (deuteriated) solutions after sonication to give rise to desired lipid:ionophore molar ratios. The concentration of the lipid used for these experiments was 18 mM.

Results

DSC Studies. DSC thermograms of DMPC and DPPC MLVs incorporated with NA, LS, and A23187 at different lipid:ionophore concentration ratios (500:1, 250:1, 100:1, and 50:1) have been recorded in the temperature range of 0–80 °C. The thermograms for free and LS incorporated DMPC MLVs are shown in Figure 2. The main chain melting transition has been observed at 23.5 °C for DMPC and 41.3 °C for DPPC, and the pretransition has been observed at 13 and 38 °C for DMPC and DPPC, respectively. The enthalpy

Table I: DSC Data for Free and Ionophore-Incorporated DMPC and DPPC MLVs

		DMPC				DPPC			
		pretransition		main transition		pretransition		main transition	
ionophore	lipid:ionophore	T_c (°C)	H (kcal·mol^{-1})	T_c (°C)	H (kcal·mol^{-1})	T_c (°C)	H (kcal·mol^{-1})	T_c (°C)	H (kcal·mol^{-1})
free		13.0	1.20	23.5	6.20	38.0	1.80	41.3	8.60
valinomycin[a]	500:1	13.0	1.00	23.5	6.20	38.0	1.60	41.3	8.50
	250:1	13.0	0.82	23.5	6.20	38.0	1.39	41.3	8.45
	100:1	13.0	0.51	23.5	6.10	38.0	1.00	41.3	8.40
	50:1	13.0	0.32	23.4	6.00	38.0	0.80	41.3	8.40
nonactin	500:1	13.0	0.95	23.5	6.20	38.0	1.42	41.3	8.50
	250:1	13.0	0.64	23.5	6.10	38.0	1.27	41.3	8.50
	100:1	13.0	0.40	23.5	6.00	38.0	0.88	41.2	8.43
	50:1	12.8	0.23	23.5	6.00	38.0	0.61	41.3	8.33
A23187	500:1	13.0	0.85	23.5	6.20	38.0	1.35	41.3	8.50
	250:1	13.0	0.56	23.5	6.00	38.1	1.05	41.3	8.30
	100:1	13.0	0.30	23.5	6.00	38.0	0.60	41.3	8.25
	50:1			23.5	5.80			41.0	8.21
lasalocid A	500:1	13.0	0.83	23.5	6.10	38.0	1.38	41.2	8.48
	250:1	13.0	0.59	23.5	6.10	38.0	0.91	41.0	8.36
	100:1	13.0	0.31	23.5	5.90	38.0	0.54	41.0	8.30
	50:1			23.5	5.90			41.0	8.16

[a] Taken from Sankaram (1983).

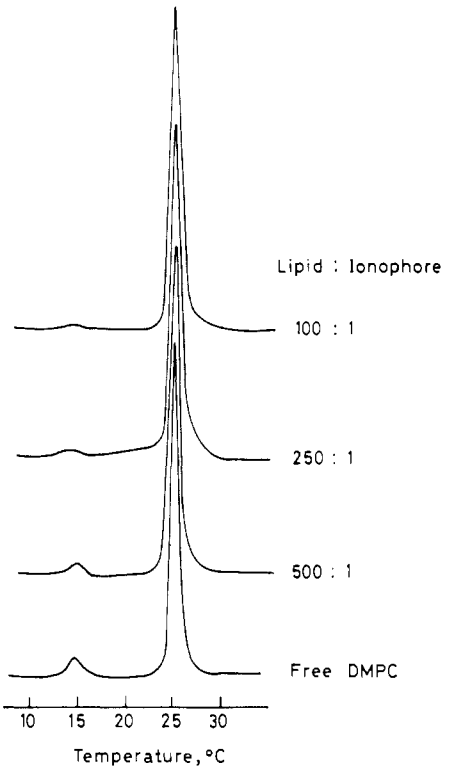

FIGURE 2: DSC thermograms of free and LS-incorporated DMPC MLVs: [DMPC] = 40 mM.

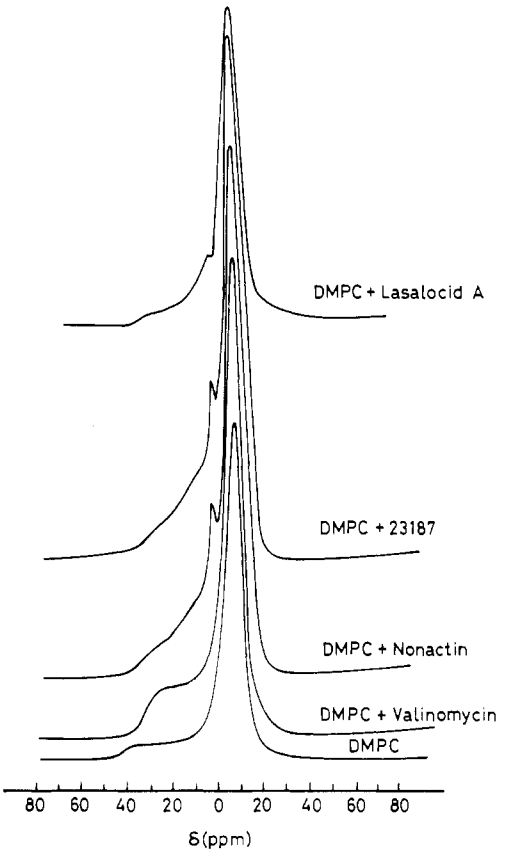

FIGURE 3: Proton-decoupled 187.5-MHz ^{31}P NMR spectra of free and ionophore-incorporated DMPC MLVs at 40 °C: [DMPC] = 60 mM (lipid:ionophore, 10:1).

values calculated for the transitions are 6.2 kcal·mol^{-1} (main transition) and 1.2 kcal·mol^{-1} (pretransition) for DMPC and 8.6 kcal·mol^{-1} (main transition) and 1.8 kcal·mol^{-1} (pretransition) for DPPC, and these values agree well with the values reported in the literature (Mabrey & Sturtevant, 1978). With increasing concentration of any of the ionophores, a broadening of the pretransition with reduction in enthalpy values has been observed. The pretransition has completely disappeared in the case of LS and A23187 at a concentration ratio of 50:1. The main transition did not show any significant changes in both DMPC and DPPC MLVs in the presence of any of the ionophores. The enthalpy values calculated for the two transitions at different concentration ratios of the ionophores for DMPC and DPPC MLVs along with the values for ionophore-free and VM-incorporated MLVs are given in Table I. It is evident from these data that although the changes in the enthalpy values at a given lipid:ionophore ratio are different for the two lipid systems, the relative effects of ionophores on the enthalpy values follow the same order irrespective of the lipid used.

^{31}P NMR Studies. The ^{31}P NMR spectra of free and ionophore-incorporated DMPC and DPPC MLVs have been recorded at 40 and 60 °C, respectively, and the spectra obtained for DMPC MLVs are shown in Figure 3. The spectra of DMPC and DPPC MLVs without any of the ionophores show axially symmetric CSA patterns with $\Delta\sigma$ values of −45.8 and −50.0 ppm, respectively. The kinds of effects produced by incorporation of the ionophores are similar in both lipid systems. The $\Delta\sigma$ values have been found to gradually decrease

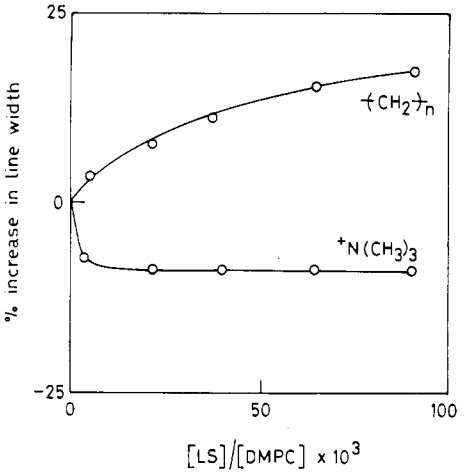

FIGURE 4: ^{1}H NMR titration graph of DMPC–LS ULVs at 40 °C: [DMPC] = 18 mM.

upon addition of VM (−35.0 and −40.0 ppm for DMPC and DPPC, respectively) in the concentration range 1000:1 to 50:1 whereas the other ionophores, viz., NA, LS, and A23187, have broadened the spectral features making it difficult to obtain reliable $\Delta\sigma$ values. However, an additional isotropic signal has been observed at 0 ppm in both lipid systems in the presence of these ionophores at lipid-ionophore ratios of <50:1.

^{1}H NMR Studies. For the ^{1}H NMR studies, ULVs have been obtained by sonication of MLVs prepared by dispersing a thin film of the lipid in D_2O as described under Materials and Methods. Ionophores have been added to these ULVs as methanolic solutions in aliquots of a few microliters (not more than 30 μL in any case) to obtain the required lipid:ionophore ratios. Control experiments have been performed by adding the same amount of methanol to a sample of the vesicle solution. Experiments have been performed with both DMPC and DPPC ULVs at 40 and 60 °C, respectively, and the line width at half-maximum of the head group choline signal and the aliphatic methylene chain signal was measured. The line width of the aliphatic methylene chain signal increased continuously for all the three ionophores (i.e., NA, LS, and A23187) whereas that of the head group choline signal decreased initially upon gradual addition of the carboxylic ionophores LS and A23187 to either DMPC or DPPC ULVs. The titration graph for line broadening of methylene and choline signals of DMPC vesicles upon LS incorporation is shown in Figure 4.

Discussion

It is evident from the DSC studies that the main chain melting transition of the MLVs is not significantly affected upon addition of any of the ionophores even at a lipid:ionophore ratio of 50:1. This suggests that none of the ionophores penetrate the hydrophobic interior of the multilamellar bilayers. This is probably due to the efficient packing of the lipid molecules in MLVs, which are "onion-like" structures. In fact, it has been shown that MLVs are more ordered than ULVs (Gaber & Peticolas, 1977).

The pretransition observed at 13 °C for DMPC and 38 °C for DPPC MLVs arises due to the intra- and intervesicular interactions among the bilayer sheets of MLVs. Any changes in these interactions would be accompanied by changes in the orientation and dynamics of the head groups with respect to the long-chain axis of the lipid molecules. The anchoring of the lipid chains to the glycerol moiety would, in turn, cause a change in the orientation of the long-chain axis with respect to the bilayer normal. All these changes have been observed for liposomes at the pretransition (Chapman, 1975; Jackobson & Papahodjopoulos, 1975; Galley et al., 1975; Janiak et al., 1976, 1979; McIntosh, 1980; Boroske & Trahms, 1983).

The effects of addition of various ionophores on the pretransitions of DMPC and DPPC MLVs are similar in the sense that all of them broaden the pretransition without changing the transition temperature. However, the efficiency of broadening, as evident from the enthalpy values given in Table I, increases in the order A23187 ≃ LS > NA > VM. Considering the differences in the chemical structures of the interacting ionophores, which are roughly of the same size, it is expected that the interaction of the neutral ionophores VM and NA would be different from that of the carboxylic ionophores LS and A23187. Broadening of the pretransition suggests that these ionophores interact with phospholipid head groups alone to modify their motional properties and/or insert themselves between neighboring head groups so as to either remove the chain tilt or permit a rather wide range of tilt angles.

The CSA patterns observed in the ^{31}P NMR spectra of phospholipid MLVs can be qualitatively explained in terms of slower overall tumbling rates for MLVs because of their large size and a fast internal rotation of the lipid head groups. Any variations in either the tumbling rates or the orientation and dynamics of the polar head groups with respect to the long-chain axis would be reflected in the observed CSA parameters and line shapes. The reduction in the $\Delta\sigma$ values observed upon addition of VM is due to insertion of the ionophore molecules between neighboring head groups resulting in removal of head group phosphate–choline interactions, leading to a relatively faster head group rotation. This is consistent with the DSC results, which show a broadening of the pretransition as a function of ionophore concentration (Sankaram & Easwaran, 1984). Addition of other ionophores has also significantly reduced the ^{31}P NMR CSA patterns up to a ratio of 50:1. Beyond this ratio, concomitant with a $\Delta\sigma$ reduction, an isotropic signal has been observed in the case of NA, LS, and A23187. This sharp signal, superimposed on the axially symmetric CSA patterns with reduced $\Delta\sigma$ and broadened σ_{11} lines, is probably due to species whose motional properties fall into the "extreme narrowing limit" on the NMR time scale. These species could be small lipid–ionophore aggregates. Several such structures have been postulated in the literature as transient species during membrane fusion and transbilayer transport processes (Verkleij et al., 1979; Verkleij, 1984) and are classified as "reverse micellar aggregates". Considering the chemical structures of these ionophores and also the DSC evidence that lipid head groups alone are involved in the lipid–ionophore interactions, it appears that these aggregates are formed by electrostatic interactions between the polar groups of the ionophores and the choline tetramethylammonium groups of lipid molecules. The ability of the polar groups of the ionophores to interact with the tetramethylammonium groups of the lipid head groups would depend upon the extent to which these polar groups are exposed on the surface of the ionophores and their accessibility to interact with lipids. The polar groups of the ionophores that coordinate to the cations in solution are mainly the carbonyl groups in the case of VM and NA and ionizable carboxyl and hydroxyl groups in the case of LS and A23187 in addition to the ether and carbonyl oxygens. It is important to note here that the carboxylic ionophores exist in anionic form under our experimental conditions (pH ~7.0) and hence are able to electrostatically interact with the cationic head groups of the lipids. Further, it has been suggested that the carboxylic ionophores

may be confined to the membrane interface in their extended conformation with the polar groups strung out to interact with lipid head groups (Painter & Pressman, 1982). These considerations suggest that the polar group exposure of the ionophores is A23187 $\simeq$ LS > NA > VM, and this order parallels the efficiency of pretransition broadening observed in the DSC studies. The absence of an isotropic signal even at a high ionophore concentration ratio in the case of VM indicates that there are no lipid–ionophore aggregates formed by this ionophore. This is probably due to the nonaccessibility of its carbonyl groups to sufficiently interact with polar head groups of the lipids due to shielding by the bulky amino acid and ester side chains (Ovchinnikov et al., 1974; Easwaran, 1985).

Detailed ^{1}H NMR line-width measurements carried out on the DMPC and DPPC ULVs with increasing concentrations of VM have revealed that it is located near the head group region in DPPC vesicles and in the hydrophobic interior of the DMPC vesicles (Sankaram & Easwaran, 1984). This observation has been interpreted on the basis that DMPC ULVs are more disordered, enabling VM to penetrate the hydrophobic core whereas in the case of DPPC ULVs, which are more ordered, VM is not able to penetrate the membrane interior. However, the present ^{1}H NMR line-width measurements show that addition of NA, LS, and A23187 broadens the methylene chain signals of both DMPC and DPPC ULVs, indicating that these ionophores penetrate the hydrophobic interior of the vesicles. The reduction in line width for choline signals of DMPC and DPPC ULVs upon addition of LS and A23187 indicates an enhanced internal motion for the lipid head groups. This is probably due to insertion of a part of the ionophore molecule between the head groups of the lipid molecules just as in the case of MLVs. The carboxylic ionophores are known to mediate the cation translocation only when their carboxylic groups are in an ionized state. Earlier studies on the interaction of LS anion with $PrCl_3$ in methanol have shown that Pr^{3+} binds to the carboxylate anion forming a tris complex (Chen & Springer, 1978). It has also been shown from ionophore conformation simulation studies at the membrane–water interface that the carboxylic ionophores will have minimum conformational energy when the carboxyl group of the molecule is in a polar environment and rest of the molecule is in a nonpolar environment (Brasseur et al., 1982; Brasseur & DeLeers, 1984). Hence, it is highly likely that while the planar aromatic rings containing the ionized carboxyl groups (salicylic acid in the case of LS and benzoxazole in the case of A23187) insert themselves between the lipid head groups leading to narrowing of the choline proton signals, the rest of the molecule is located in the membrane interior broadening the methylene chain signals by way of restricting their motional freedom.

Haynes et al. (1980) and Kolber and Haynes (1981) have used the intrinsic fluorescence of LS and A23187 to study the location of these ionophores in the membrane. Both LS and A23187 were found to be bound to the membrane in the head group region. Their interactions were strongly dependent on the lipid head group, signifying head group–ionophore interactions. In addition, LS was found to exist in two different membrane-bound forms having two different lifetimes. Our experiments were unable to detect such differences. The two species observed by them could differ in the extent of burial within the membrane and/or be oriented differently in the membrane.

In conclusion, the results presented here indicate that all the three ionophores, namely, NA, LS, and A23187, interact with the polar head groups of the lipids in MLVs and that they penetrate the hydrophobic interior of the ULVs. In the case of carboxylic ionophores, LS and A23187, ^{1}H NMR line-width measurements have shown that while their aromatic rings are possibly inserted between the head groups of lipid molecules in DMPC and DPPC ULVs, the rest of the molecule may be buried in the hydrophobic core of the model membranes. Further studies aimed at elucidating the conformations of these ionophores in model membranes by circular dichroism and fluorescence techniques are in progress. The DSC studies indicate that these ionophores affect only the pretransition that has been associated with the orientation and dynamics of the lipid head groups. Another interesting outcome of the present studies is that NA, LS, and A23187 form lipid–ionophore aggregates when incorporated into the MLVs at high concentration ratios as evidenced by ^{31}P NMR studies. However, the exact morphology and structure of such aggregates is hitherto unknown and requires further probing in this direction.

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Registry No. DMPC, 18194-24-6; DPPC, 63-89-8; NA, 6833-84-7; LS, 25999-31-9; A23187, 52665-69-7.

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Resonance Raman Spectroscopy of Octopus Rhodopsin and Its Photoproducts†

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ABSTRACT: We report here the resonance Raman spectra of octopus rhodopsin and its photoproducts, bathorhodopsin and acid metarhodopsin. These studies were undertaken in order to make comparisons with the well-studied bovine pigments, so as to understand the similarities and the differences in pigment structure and photochemical processes between vertebrates and invertebrates. The flow method was used to obtain the Raman spectrum of rhodopsin at 13 °C. The bathorhodopsin spectrum was obtained by computer subtraction of the spectra containing different photostationary mixtures of rhodopsin, isorhodopsin, hypsorhodopsin, and bathorhodopsin, obtained at 12 K using the pump–probe technique and from measurements at 80 K. Like their bovine counterparts, the Schiff base vibrational mode appears at ~1660 cm^{-1} in octopus rhodopsin and the photoproducts, bathorhodopsin and acid metarhodopsin, suggesting a protonated Schiff base linkage between the chromophore and the protein. Differences between the Raman spectra of octopus rhodopsin and bathorhodopsin indicate that the formation of bathorhodopsin is associated with chromophore isomerization. This inference is substantiated by the chromophore chemical extraction data which show that, like the bovine system, octopus rhodopsin is an 11-cis pigment, while the photoproducts contain an all-trans pigment, in agreement with previous work. The octopus rhodopsin and bathorhodopsin spectra show marked differences from their bovine counterparts in other respects, however. The differences are most dramatic in the structure-sensitive fingerprint and the HOOP regions. Thus, it appears that although the two species differ in the specific nature of the chromophore–protein interactions, the general process of visual transduction is the same.

It is generally believed that bathorhodopsin is the primary photoproduct of the absorption of a photon by the visual pigment rhodopsin (Yoshizawa & Wald, 1963). Bathorhodopsin then undergoes a series of thermal transformations through a sequence of intermediates (bleaching), eventually triggering visual excitation. The sequence of changes has been studied both by kinetic methods and by trapping the various intermediates at low temperatures [reviewed by Yoshizawa (1972) and Ottolenghi (1980)]. The rhodopsin to bathorhodopsin transition is of central importance in understanding the photophysics of vision, since it involves the conversion of the photon energy to the chemical energy responsible for visual

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