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Structural Reorganizations in Lipid Bilayer Systems: Effect of Hydration and Sterol Addition on Raman Spectra of Dipalmitoylphosphatidylcholine Multilayers†

S. Fowler Bush,‡ Ralph G. Adams, and Ira W. Levin*

ABSTRACT: Vibrational Raman spectroscopy was used to investigate the conformational behavior of dipalmitoylphosphatidylcholine (DPPC) bilayers perturbed by cholesterol and water, two membrane components whose lipid interactions involve different regions of the bilayer matrix. Upon the addition of cholesterol, an intrinsic membrane constituent, to an anhydrous bilayer in concentrations varying from 7 to 30 mol %, modifications in lateral chain interactions were observed by monitoring spectral changes in the methylene C–H stretching and the CH_2 deformation regions. The perturbation in the 1460-cm^{-1} region was not spectroscopically observed until after the addition of 7 mol % of the sterol. Although chain–chain interactions are altered, no additional trans/gauche isomerization is developed along the hydrocarbon chains. Water, a peripheral bilayer component, was added to the multilayer assembly in the hydration range of 0.3 to ~4 molecules of water per lipid molecule. Vibrational spectra characteristic of motions in the head-group, interfacial, and acyl chain regions of the lipid bilayer were observed. These data indicate that hydration confers a mobility to the head-group, glycerol, and carbonyl moieties. Shifts in the CN symmetric and PO_2^- antisymmetric stretching modes, occurring on the addition of approximately four molecules of water, indicate a conformational rearrangement within the polar head group. After approximately four molecules of water are added to the DPPC system, the spectral features of the gel system [70% (w/w) water] indicate that no further head-group changes nor increases in either acyl chain trans/gauche or lattice disorder arise on further hydration.

Since the conformational and dynamical behavior of biological membranes reflects the concerted interactions of both intrinsic and extrinsic bilayer components, the origins of structural rearrangements within the lipid matrix are often difficult to resolve in liposomal systems. In particular, effects from either lipid head-group or acyl-chain perturbations may be propagated throughout the molecule, leading to structural reorganization of the entire phospholipid system. Of the wide variety of physical techniques available for investigating bilayer interactions and lipid rearrangements, vibrational Raman spectroscopy offers a sensitive analytical means both for monitoring inter- and intramolecular membrane disturbances and for identifying the region of the lipid molecule responding to the perturbation [see, for example, Wallach et al. (1979) and references cited therein]. The Raman scattering effect provides spectral features whose frequencies correspond to molecular vibrations which are assigned to motions within structurally distinct areas of the phospholipid molecule: for example, the lipid polar head-group region (symmetric C–N and PO_4^- stretching modes), the interface region (the inequivalent C=O stretching modes and the glycerol backbone C–C stretching mode), and the hydrophobic hydrocarbon chain region (CH_2 stretching, twisting, and deformation modes and the skeletal C–C stretching modes). By following changes in the spectral frequencies and intensities of these vibrational transitions as a function of either temperature or addition of a membrane component, one is able to assess the conformational modifications and lattice packing variations assumed by the lipid assembly under given bilayer conditions.

Of particular interest in the present study is the comparison between two membrane components, cholesterol and water, whose bilayer interactions involve different molecuar sites within the phospholipid assembly. By monitoring the Raman spectrum of an anhydrous dipalmitoylphosphatidylcholine (DPPC) lattice perturbed by quantitatively adding cholesterol and water, either singly or together, we are able to survey simultaneously the various bilayer events occurring within the three structurally distinct regions of the lipid molecule. For the DPPC–water system we follow specifically the lipid inter- and intramolecular changes induced by adding 0.3 to ~4 molecules of water per lipid molecule. Although we emphasize in this study the changes in relatively rigid lipid lattices defined by low water contents, we stress the important point that the head-group conformation, structural arrangements about the fatty acid carbonyl groups, and the trans/gauche chain disorder (at a given temperature) are developed by approximately the first four water molecules of hydration and do not change appreciably under conditions of excess water [70% (w/w)].

A relatively detailed view regarding the structural and motional requirements of cholesterol in lipid bilayers has been generated from a wide variety of biochemical and physical studies emphasizing, for example, the modification of mem-

† From the Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205. *Received March 26, 1980.*
‡ On sabbatical leave from the Department of Chemistry, University of North Carolina at Charlotte, Charlotte, NC.

brane function by sterols (Lala et al., 1978, 1979; Demel & Kruyff, 1976) and the characterization of sterol–lipid interactions by spectroscopic (Jacobs & Oldfield, 1979; Brown & Sellig, 1978; Oldfield et al., 1978; S. F. Bush, H. Levin, and I. W. Levin, unpublished experiments), diffraction (Pearson & Pascher, 1979; Calhoun & Shipley, 1979; Worcester & Franks, 1976; Elder et al., 1977), and model-building (Huang, 1977a,b; Brockerhoff, 1974) techniques. The essential structural requirements for lipid–sterol interaction involve a planar (trans-fused rings) steroid nucleus, a lengthy aliphatic side chain (Craig et al., 1977; Suckling & Boyd, 1975), and perhaps a 3β-OH group (Lala et al., 1979). The sterol component in both biological and model membranes occupies a position normal to the bilayer plane with its ring system juxtaposed to the upper C(1)–C(9) half of the lipid acyl chains. The 3β-OH group extends into the bilayer interfacial region, while the aliphatic side chain aligns along the lower portion of the lipid acyl chains.

A number of lipid–cholesterol models have pursued the attractive notion of hydrogen bonding between the sterol OH group and a suitable proton acceptor. ^{31}P NMR studies (Cullis et al., 1975; Yeagle et al., 1975), however, preclude the phosphate moiety as a possible hydrogen-bonding site. The only other suitable hydrogen-bond acceptor sites would involve the acyl chain carbonyl oxygen atoms with both the 1-chain (Huang, 1977a,b) and 2-chain carbonyl groups having been separately proposed (Brockerhoff, 1974; Tirri et al., 1977).

The effects of water on phospholipid bilayers in the absence of cholesterol have been investigated by nuclear magnetic resonance (Griffin, 1976), X-ray (Pearson & Pascher, 1979) and neutron diffraction (Büldt et al., 1979) techniques. The time-averaged X-ray structure locates the water molecules within the polar head-group region for a DMPC sample containing two water molecules of hydration (Pearson & Pascher, 1979). The authors position one water molecule between the phosphate groups of adjacent lipid molecules, which insulates the anionic phosphate moieties, and propose an infinite hydrogen-bonded phosphate (A)–water–phosphate (B)–water ribbon. The remaining water of hydration was proposed to hydrogen bond to a phosphate oxygen on one side of the bilayer interface and to an oxygen atom of a water molecule in an adjacent ribbon across the interface. A bonding scheme such as this would provide stability in a direction perpendicular to the ribbon linkage. Although the ^{31}P NMR study does not conclusively rule out hydrogen bonding to the phosphate group, a plausible argument is made that water [for samples from 6 to 10% (w/w) H_2O] induces a rotational motion of the PO_4^- group (Griffin, 1976). A recent neutron diffraction study of DPPC in the gel state concludes that at 6% (w/w) water a large proportion of the water molecules enters between the head groups but penetrates no further than the uppermost C(3) carbon of the glycerol backbone (Büldt et al., 1979). An increase in water content to 25% (w/w) for the gel state results in greater numbers of water molecules penetrating the head-group region, a condition which further reduces electrostatic interactions between the charged polar regions. No conclusions are reached, however, regarding the level of penetration of water in the liquid-crystalline state.

Experimental Procedures

High-purity samples (99% or better) 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine (DPPC) and cholesterol were purchased from Sigma Chemical Co. and used without further purification. All samples, with and without cholesterol, were first crystallized from chloroform and then evacuated at 10^{-5} torr for at least 48 h to ensure complete removal of water as demonstrated by the absence of water absorptions in the 3400- and 1620-cm^{-1} regions of the infrared spectra. The anhydrous samples were transferred under a nitrogen gas atmosphere to dried capillary tubes and sealed. Hydrated samples were obtained by exposing the anhydrous materials to a nitrogen atmosphere at 95% humidity in a drybox for up to 5.5 h. Samples were removed at various times. After determining the degree of hydration gravimetrically, the samples were sealed in dry capillary tubes.

Table I: Frequency (cm^{-1}) and Peak Height Intensity (I_a/I_b)[a] Comparisons for Anhydrous Dipalmitoylphosphatidylcholine (DPPC)–Cholesterol Bilayers

	mol % cholesterol =				
	0	7	14	20	30
C–N stretching frequency (cm^{-1})	710	710	710	710	710
I_{1720}/I_{1738}	0.88	0.89	0.86	0.85	0.84
I_{1128}/I_{1062}	0.91	0.93	0.92	0.93	0.92
I_{2937}/I_{2880}	0.37	0.38	0.39	0.42	0.41
I_{1460}/I_{1436}	0.95	0.95	0.87	0.88	0.79

[a] Contributions from cholesterol vibrational transitions have been subtracted from spectra of the various DPPC–cholesterol mixtures.

Vibrational Raman spectra were recorded with a Spex Ramalog 6 spectrometer, equipped with plane holographic gratings, with a spectral resolution of 5 cm^{-1}. Excitation radiation at the sample of ~100 mW at 514.5 nm was provided by a Coherent Model CR-3 argon ion laser. Spectral frequencies, calibrated with atomic argon lines, are reported to ±2 cm^{-1}. Raman data were acquired with a Nicolet NIC-1180 data system interfaced to the spectrometer. Since the carbonyl spectral features exhibited weak Raman scattering, spectra displayed in Figures 1–7 resulted from signal averaging 5–30 scans at a 1 cm^{-1} s^{-1} scan rate. Samples were maintained at ambient temperatures (~27 °C) during spectral scans.

Deconvoluted Raman spectra were resolved by using standard software provided with the NIC-1180 data system. Spectral subtractions of cholesterol backgrounds were carried out for the lipid–sterol samples in the CH_2 deformation region (1350–1550 cm^{-1}), the C–H stretching region (2800–3100 cm^{-1}), and the C–C stretching region (1050–1150 cm^{-1}). Cholesterol spectral contribution in the C–N region was negligible. The relative amounts of a standard cholesterol Raman spectrum to be subtracted were determined by nulling the C=C spectral feature at 1671 cm^{-1} of cholesterol in each sterol spectrum.

Peak height intensity ratios presented in Tables I and II were determined from expanded spectral traces of the signal-averaged spectra and reflect an accuracy of ±5%. No computer smoothing routines were applied to the signal-averaged spectra.

Results and Discussion

In the present studies, the perturbations to the lipid matrix resulting from addition of cholesterol, an intrinsic membrane component, and water, an extrinsic component, were examined separately. Vibrational frequencies and intensities characteristic of the lipid interface, head-group, and hydrocarbon chain regions provide structural probes for the bilayer system. Two series of experiments were performed. In the first set cholesterol alone was added to anhydrous DPPC bilayers in 7–30 mol % increments (Table I; Figures 1, 4, and 6); while in the second series, water was quantitatively added to the bilayer (Table II; Figures 2, 3, 5, and 7).

A final series of experiments was designed in order to assess simultaneous bilayer effects of adding both cholesterol and

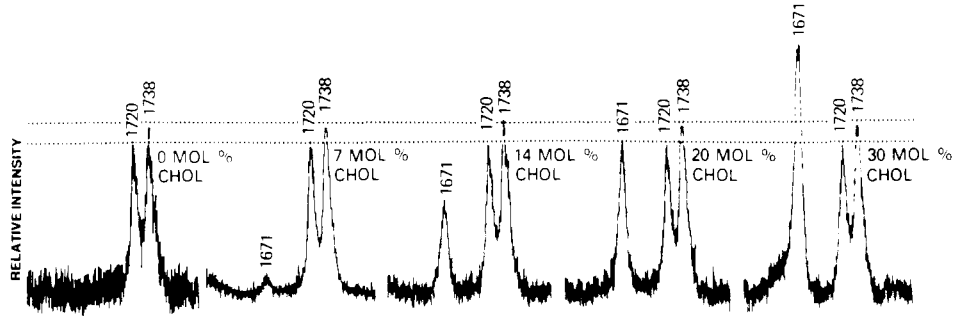

FIGURE 1: Spectra in the carbonyl stretching mode region of anhydrous DPPC with varying amounts of cholesterol. The 1671-cm^{-1} feature represents a contribution from the cholesterol double bond stretching mode frequency. Spectra are normalized to the 1738-cm^{-1} feature of DPPC.

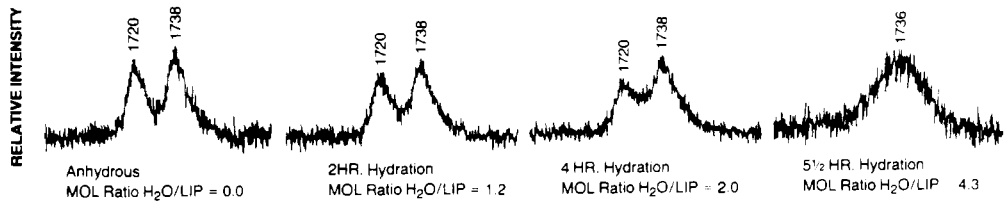

FIGURE 2: Spectra in the carbonyl stretching mode region of hydrated DPPC.

Table II: Frequency (cm^{-1}) and Peak Height Intensity (I) Comparisons for Hydrated Dipalmitoylphosphatidylcholine (DPPC) Bilayers

	mol ratio of water/DPPC[a] =					
	0	0.3	0.8	1.2	2.0	4.3
C-N stretching frequency (cm^{-1})	711	711	711	711	711	717
I_{1720}/I_{1738}	0.88	0.88	0.85	0.86	0.71	*b*
I_{1128}/I_{1062}	0.90	0.91	0.89	0.91	0.87	0.81
I_{2937}/I_{2880}	0.36	0.34	0.33	0.34	0.34	0.34
I_{1460}/I_{1436}	0.95	0.95	0.93	0.91	0.84	0.77

[a] Mole ratio of water/DPPC, hydration time in hours, and weight percent water are given as follows: 0, 0, 0; 0.3, 0.5, 0.7; 0.8, 1.0, 1.9; 1.2, 2.0, 2.8; 2.0, 4.0, 4.6; 4.3, 5.5, 9.6. [b] Vibrational transitions coalesce.

water to the bilayer; thus, spectra were recorded of DPPC with 14 mol % incorporated cholesterol at the same hydration levels as shown in Table II. Intensity ratios for this specific series of spectra are not presented since the data for the three-component system compare closely to the two-component water–DPPC system. Under identical conditions, the sterol-containing lipid samples hydrated at the same rate as those without cholesterol.

(A) Interfacial Region: C=O Stretching Modes (1600–1800 cm^{-1}). In a recent study (S. F. Bush, H. Levin, and I. W. Levin, unpublished experiments) we assigned the two spectral features observed at 1738 and 1721 cm^{-1} in anhydrous DPPC to the carbonyl stretching modes associated with the lipid 1- and 2-chain positions, respectively. Although we discussed the cumulative effects of both cholesterol and water upon the carbonyl modes, the C=O spectral region is considered again here in order to include the results of DPPC hydration in the absence of cholesterol.

Figure 1 shows that the incorporation of up to 30 mol % cholesterol has virtually no effect upon the carbonyl stretching mode intensities or frequencies. Examination of Table I indicates that the I_{1720}/I_{1738} ratio for the carbonyl features remains constant, 0.86 ± 0.02, for sterol addition. Since cholesterol (up to 30 mol %) perturbs neither the 1- nor 2-chain carbonyl stretching modes, hydrogen bonding between the 3β-OH and acyl carbonyl groups is clearly precluded in the anhydrous bilayer (S. F. Bush, H. Levin, and I. W. Levin, unpublished experiments). In this context we note that neutron diffraction studies for samples of 10–20% (w/w) water content place the hydroxyl group at the edge of the polar region (Worcester & Franks, 1976).

The hydration experiments shown in Figure 2 demonstrate a more dramatic change in the 1720–1740-cm^{-1} region than that observed with cholesterol. As shown in Table II the I_{1720}/I_{1738} ratio decreases from 0.88 for anhydrous DPPC to a value of 0.71 when two molecules of water are added. When approximately four molecules of water are added, the 1720- and 1738-cm^{-1} vibrational transitions coalesce, indicating that the second pair of water molecules affects the bilayer in a somewhat different manner. The intensity changes observed for the addition of up to two molecules of water are attributed to a decrease in the dielectric constant of the medium that occurs on separating the polar head groups with the insertion of water molecules. Since the 2-chain carbonyl group is closer to the phosphorylcholine group (Pearson & Pascher, 1979; Elder et al., 1977; Hitchcock et al., 1974) its intensity is more readily affected by changes in charge distribution involving the polar head-group regions. This positioning of the carbonyl group accounts for the more rapid intensity reduction of the 2-chain C=O stretching mode at 1720 cm^{-1} as compared to that for the 1-chain C=O stretching mode at 1738 cm^{-1}. This general pattern for the changes in the Raman intensities is consistent with the recent X-ray study (Pearson & Pascher, 1979) in which the first two water molecules of hydration, located within the head-group phosphate region, apparently act both to separate and to shield the phosphate charges.

Table II lists in greater detail the small changes in the I_{1720}/I_{1738} intensity ratio as up to one water per lipid molecule is added to the bilayer. This ratio significantly decreases as the second water molecule per lipid is incorporated, and, as noted above, the spectral features coalesce when the addition totals four molecules of water. In a previous analysis involving deconvoluted spectra, it was noted that the intensity of the 1720-cm^{-1} C=O stretching mode component decreases while a higher frequency feature at ~1731 cm^{-1} increases in intensity. This spectral behavior is clearly not that expected for strong hydrogen-bond formation at a carbonyl site (S. F. Bush, H. Levin, and I. W. Levin, unpublished experiments). In summary at this point, Raman scattering provides spectral evidence for a distinction in the effects on the bilayer between

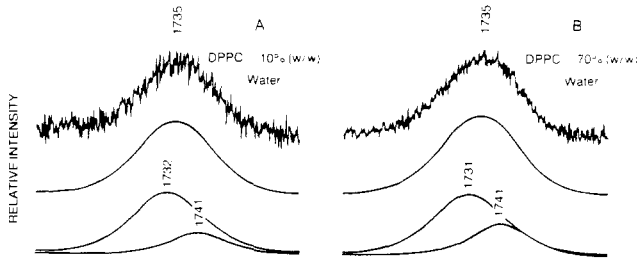

FIGURE 3: Deconvoluted spectra in the carbonyl stretching mode region for hydrated DPPC for (A) 10% (w/w) water and (B) 70% (w/w) water at 25 °C. For each panel the top trace represents the observed spectrum, the middle trace represents the calculated spectrum, and the bottom trace represents the deconvoluted spectrum.

the addition of the first and second water molecules of hydration. These separate spectral effects may be related to the X-ray analysis which delineates different bonding schemes for each of the two waters of hydration (Pearson & Pascher, 1979).

After the addition of approximately four molecules of water, no further frequency, intensity, or contour changes are observed in the carbonyl stretching mode region with increased hydration. Figure 3 displays deconvoluted spectra for the 1710–1760-cm^{-1} interval for DPPC at 28 °C with 10% (w/w) water, approximately four molecules of water per lipid, and with 70% (w/w) water at 25 °C. The upward shift in frequency from 1720 cm^{-1} for the anhydrous and partially hydrated states to ~1735 cm^{-1} is attributed to changes in the 2 chain as it becomes more conformationally equivalent to the 1 chain (S. F. Bush, H. Levin, and I. W. Levin, unpublished experiments). The broadening and merging of the spectral features in the carbonyl region reflect not only chain rotations but probably a rotational mobility in the glycerol backbone. This motional behavior assumed by the lipid in the gel state is first reached, however, at hydration levels of only approximately four molecules of water per lipid.

Finally, spectra recorded of 14 mol % cholesterol–DPPC bilayers at various levels of hydration indicate a synergistic effect for water and cholesterol. That is, the I_{1720}/I_{1740} ratio decreases more rapidly as hydration proceeds with cholesterol present in the lipid matrix. For 14 mol % cholesterol the ratio decreased from 0.86 to 0.72 as the first water molecule was added, while, in the absence of cholesterol, no change in the I_{1720}/I_{1738} ratio was detected for the same degree of hydration. This result suggests that cholesterol may alter or participate in the binding scheme of the water network implied by the time-averaged X-ray structure. Alternatively, cholesterol, acting as a spacer molecule, may increase the rate at which the dielectric properties of the bilayer are affected when water is added.

(B) Head-Group Region: C–N Stretching (700–730 cm^{-1}) and PO_2^- Antisymmetric Stretching (1250 cm^{-1}) Modes. The head-group C–N stretching mode at ~710–717 cm^{-1} and the nonester oxygen PO_2^- antisymmetric stretching mode at 1253 cm^{-1} in the Raman spectrum of DPPC have been previously assigned and discussed [see, for example, Bunow & Levin (1977a) and Spiker & Levin (1975)].

As shown in Figure 4A and Table I, the 710-cm^{-1} symmetric C–N stretching mode, characteristic of the choline moiety, remains invariant as the cholesterol content is increased from 0 to 30 mol %. The constancy of both the intensity and the frequency of this mode indicates that no conformational changes occur within the head group of the DPPC molecule when cholesterol spacers are inserted within the bilayer. The ~700-cm^{-1} feature, observed as a shoulder in Figure 4A in spectra of higher cholesterol concentrations, is characteristic of a vibrational mode of cholesterol.

Examination of the hydration data in both Table II and Figure 5A shows an abrupt shift to higher frequencies in the C–N stretching mode frequency from 711 to 717 cm^{-1} as the second pair of water molecules is added. We compare these effects to the hydration effects for the 1253-cm^{-1} PO_2^- antisymmetric stretching mode (spectra not shown). Upon the addition of two molecules of water, the vibrational frequency remains at 1253 cm^{-1}, the Raman value characteristic of an anhydrous DPPC sample. A slight decrease in band intensity

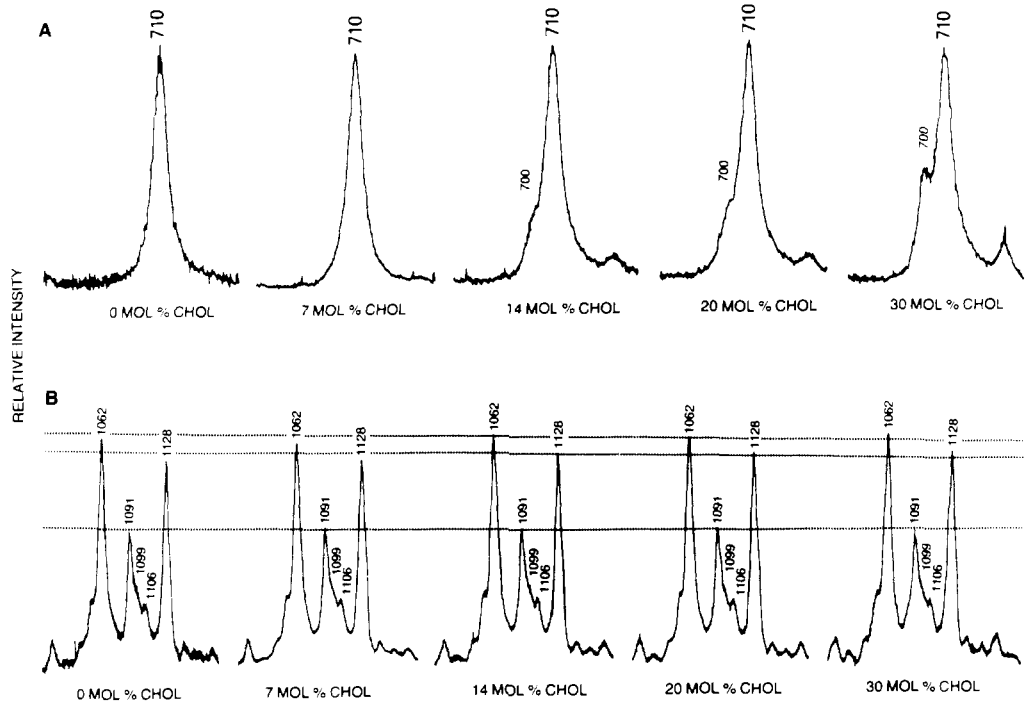

FIGURE 4: Spectra of anhydrous DPPC with varying amounts of cholesterol in (A) the C–N stretching mode region and (B) the C–C stretching mode region. In (B) the spectra are normalized to the 1062-cm^{-1} feature.

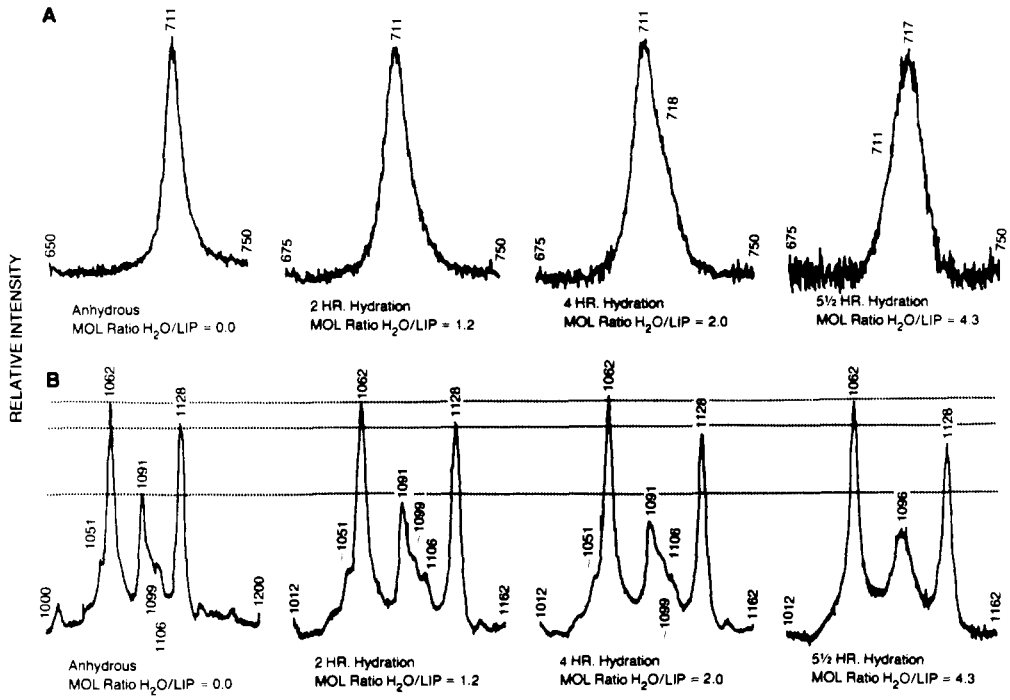

FIGURE 5: Spectra of hydrated DPPC in (A) the C–N stretching mode region and (B) the C–C stretching mode region. In (B) the spectra are normalized to the 1062-cm^{-1} feature.

occurs, however, on the addition of the second water molecule. As the third and fourth water molecules are added to the bilayer, the frequency shifts from 1253 to 1242 cm^{-1}. (These data, along with infrared spectra, will be discussed elsewhere in further detail.)

The Raman spectral behavior of the phosphate vibration during the addition of the first two water molecules is consistent with the ^{31}P NMR study (Griffin, 1976) which associates changes in spectral signals of DPPC samples of low water content with an onset of rotation of the PO_4^- group rather than the formation of hydrogen bonds. Thus, there is no spectral evidence for a shift in the phosphate frequency to lower wavenumbers, and we conclude that the first two water molecules are not involved in strong hydrogen bonding to the phosphate oxygen atoms. Although the ^{31}P NMR results for 10% (w/w) water (four molecules of water per lipid molecule) indicate a motional averaging of the PO_4^- structural unit, the vibrational shift to lower frequencies of $\sim$11 cm^{-1} on the addition of four molecules of water is suggestive of a hydrogen-bonding effect. However, the concomitant increase of the choline C–N stretching mode with four molecules of water gives greater weight to an interpretation involving a structural rearrangement of the entire head group. Further hydration beyond 10% (w/w) water to at least 70% (w/w) does not significantly affect either the 717- or 1243-cm^{-1} features, emphasizing the ^{31}P NMR conclusion that the PO_4^- (and probably the entire polar head group) conformation is determined by the first four or five waters of hydration (Griffin, 1976). (The 717-cm^{-1} feature does exhibit a slight shift of $\sim$1 cm^{-1} to 716 cm^{-1} for DPPC samples 70% (w/w) water, which may reflect a slight alteration in a bond length or bond angle involving the nitrogen atom.)

The 700 cm^{-1} region spectral data for hydrating DPPC bilayers containing 14 mol % cholesterol are identical with the effects shown in Table II for samples without cholesterol. Thus, the hydration layers about the head group are apparently not perturbed by the sterol 3β-OH group.

(*C*) *Hydrocarbon Chain Region: C–C Stretching Modes (1050–1150 cm^{-1}).* Although the 1050–1150-cm^{-1} spectral interval is vibrationally congested, three Raman transitions for DPPC bilayers (Figure 4B) at 1062, 1091, and 1128 cm^{-1} have been assigned to acyl-chain all-trans C–C stretching modes [see, for example, Spiker & Levin (1975) and Bunow & Levin (1977a)]. The intense 1062-cm^{-1} feature is both chain-length and temperature insensitive, whereas the weaker 1091- and intense 1130-cm^{-1} lines are functions of both temperature and chain length. Weaker features centered at $\sim$1099 and 1106 cm^{-1} are assigned to C–C stretching modes of the choline head group and of the glycerol moiety, respectively, on the basis of spectral comparisons with model systems (unpublished observations on dipalmitoylglycerol, glycerophosphorylcholine, and highly ordered L-α-DPPC). Gauche conformers occurring along the hydrocarbon chains are reflected by an increase in intensity in the $\sim$1085–1090-cm^{-1} interval and by a decrease in intensity in the 1128-cm^{-1} transition. The PO_4^- nonester oxygen symmetric stretching mode contributes weakly to the $\sim$1082-cm^{-1} contour. The glycerol C–O stretching modes are assigned to the 1051-cm^{-1} feature.

Examination of the 1050–1150-cm^{-1} C–C stretching mode region in Figure 4B indicates that cholesterol alters neither the intensities nor the frequencies of the spectral transitions in this interval. Specifically, since no spectral changes appear in the 1130-cm^{-1} all-trans marker, as demonstrated by the constant I_{1128}/I_{1062} ratio in Table I, or in the 1085–1090-cm^{-1} gauche marker, we conclude that no intrachain disorder results from the addition of up to 30% cholesterol at ambient temperatures.

In contrast, as shown in Figure 5B and Table II, a decrease in the I_{1128}/I_{1062} ratio and an increase in the intensity of the 1085–1090-cm^{-1} region occurs with the addition of two molecules of water. A dramatic decrease in the I_{1128}/I_{1062} marker occurs after the addition of two molecules of water, as displayed in the spectrum (Figure 5B) of a sample with a H_2O/lipid ratio of $\sim$4. The 10% decrease in the 1128-cm^{-1} all-trans marker and a filling in of intensity in the 1085–1090-cm^{-1} region indicate an increase in intrachain disorder stemming from the formation of gauche conformers within the

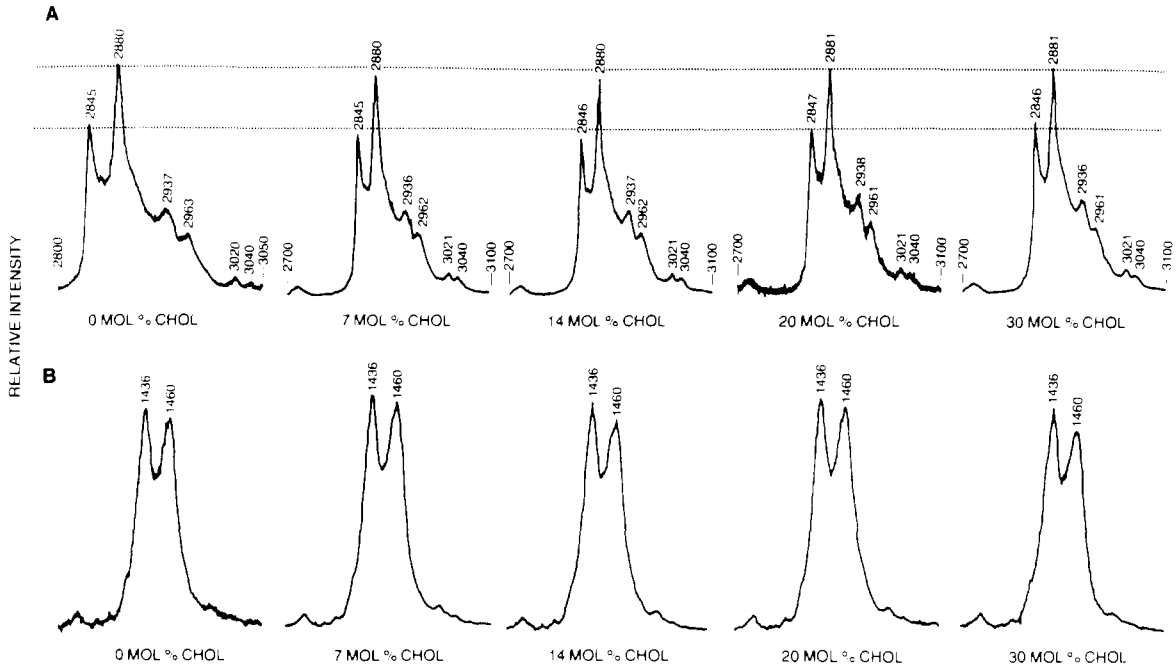

FIGURE 6: Spectra of anhydrous DPPC with varying amounts of cholesterol in (A) the C–H stretching mode region and (B) the CH_2 deformation mode region. Spectra are normalized to the 1436- and 2845-cm^{-1} features.

hydrophobic portion of the bilayer. The increased molecular mobility for the head-group and interfacial regions caused by the addition of water apparently allows an expansion of the lipid lattice which favors the formation of gauche conformers and induces the loss of bilayer crystallinity.

The spectra in Figure 5B indicate that for two waters of hydration the 1099-cm^{-1} feature, assigned to a head-group C–C mode, begins to broaden. The glycerol C–O stretching mode at ~1051 cm^{-1} broadens and essentially disappears upon addition of approximately four molecules of water per lipid. These line-broadening effects correlate with the above discussion concerning the C–N, PO_2^-, and C=O stretching mode behavior which indicated an enhanced mobility for the head-group and glycerol moieties.

Spectral effects in the 1050–1150-cm^{-1} interval for hydrated DPPC samples containing 14 mol % cholesterol parallel the spectral behavior of pure DPPC bilayers. Thus, under these conditions the sterol apparently neither enhances nor diminishes the effects of water on intrachain disorder.

(D) Hydrocarbon Chain Region: C–H Stretching (2800–3100 cm^{-1}) and CH_2 Deformation (1400–1500 cm^{-1}) Modes. The C–H stretching and CH_2 deformation spectral regions have been used to monitor changes in the lipid-chain lateral packing characteristics in gel and liquid-crystalline bilayer assemblies [see, for example, Yellin & Levin (1977), Gaber & Peticolas (1977), and Spiker & Levin (1976)]. Although curve resolution in the methylene and methyl 2800–3100-cm^{-1} C–H stretching region is exceedingly difficult and perhaps ambiguous, the various bilayer intra- and intermolecular disordering processes may be conveniently monitored by changes in the relative peak height intensity ratios for the 2845-, 2880-, and 2937-cm^{-1} features (Yellin & Levin, 1977). These spectral transitions are assigned respectively to the methylene C–H symmetric stretching, methylene C–H asymmetric stretching, and, in part, the chain terminal methyl C–H symmetric stretching modes. As the bilayer undergoes intramolecular chain disorder, the intensity beneath the 2937-cm^{-1} feature increases as a result of the appearance of an underlying manifold of infrared-active methylene asymmetric stretching modes (Bunow & Levin, 1977b). Table I and Figure 6A present the I_{2937}/I_{2880} peak height intensity ratios for the addition of cholesterol to the anhydrous DPPC bilayer. The slight but definite increase in this ratio demonstrates a gradual intermolecular disordering of the lipid lattice as more sterol intercalates between the acyl chains. Since the I_{1128}/I_{1062} ratio (Table I) indicates no increase in gauche conformers on cholesterol addition, the C–H stretching region for this system reflects only the change in lateral chain–chain interactions.

The spectral behavior of the CH_2 1450-cm^{-1} deformation modes confirms the lattice disorder implied by the C–H stretching region parameters. In general, as a pure gel, DPPC for example, approaches its gel to liquid-crystalline phase transition temperature and undergoes lattice disorder, the 1460-cm^{-1} feature, reflecting the high-frequency component of the CH_2 deformation doublet, decreases in intensity and broadens into a shoulder of the 1436-cm^{-1} component (Yellin & Levin, 1977). For the anhydrous DPPC–cholesterol system treated here, the spectral similarities are analogous. Spectra in Figure 6B and the I_{1460}/I_{1436} peak-height ratios in Table I demonstrate cholesterol's participation in decreasing lipid interchain interactions. The perturbation is not spectroscopically observed, however, until after the addition of 7 mol % of the sterol. A comparison of the I_{2937}/I_{2880} and I_{1460}/I_{1436} ratios in Table I indicates that the CH_2 deformation region parameters reflect with more sensitivity the small changes in lateral interchain order.

On bilayer hydration (with one, two, and approximately four molecules of water per lipid molecule) the I_{2935}/I_{2880} peak height intensity ratios remain constant, although the I_{1460}/I_{1436} ratios show a definite trend toward lattice disorder (Table II and Figure 7). As in the case of the anhydrous DPPC plus cholesterol system, the 1450-cm^{-1} CH_2 deformation region again appears more sensitive to changes in lattice packing than the 2900-cm^{-1} C–H stretching mode region. However, the two features at 3020 and 3040 cm^{-1}, assigned to the choline CH_3 asymmetric stretching modes (Spiker & Levin, 1976), broaden and finally merge as the second pair of water molecules is added to the lipid bilayer (Figure 7A). The coalescence of these spectral features is again consistent with an increase in mobility within the head-group region which occurs simulta-

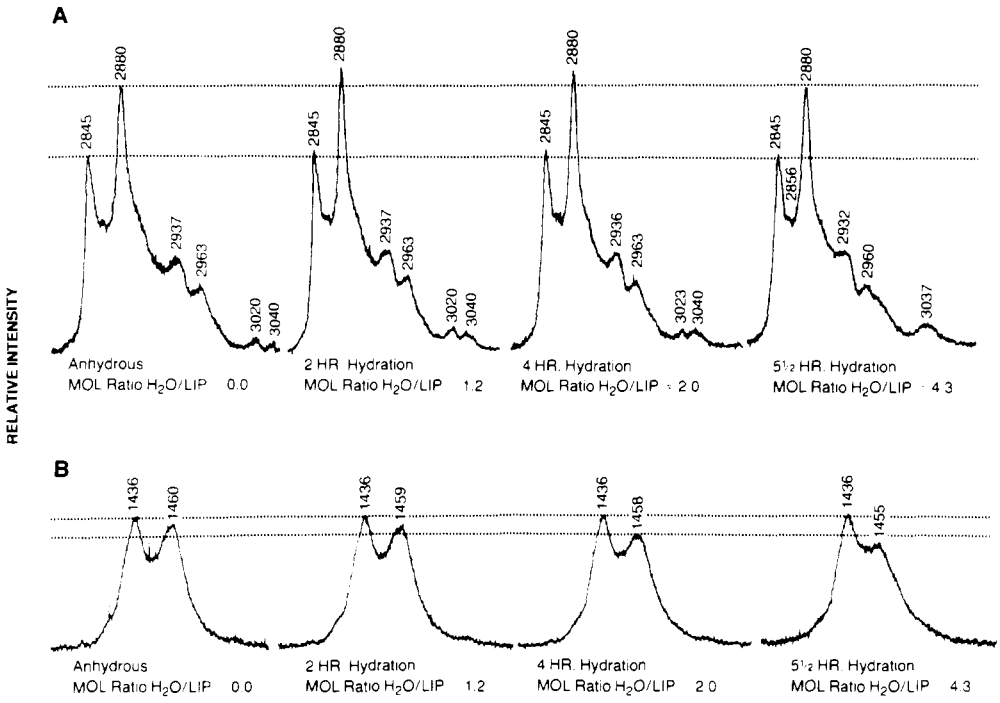

FIGURE 7: Spectra of hydrated DPPC in (A) the C–H stretching mode region and (B) the CH_2 deformation mode region. Spectra normalized to the 2845- amd 1436-cm^{-1} features.

neously with the gradual trans/gauche disordering of the hydrocarbon chains in the hydrophobic portion of the bilayer. Finally, addition of water to DPPC samples containing 14 mol % cholesterol produces spectral effects analogous to those observed in spectra of the hydrated samples of pure DPPC.

Conclusion

Observation of vibrational Raman spectral frequencies and intensities enables one both to monitor intramolecular changes occurring within the three structurally distinct regions of the phospholipid molecule and to follow alterations in lattice packing characteristics. On addition of cholesterol, an integral membrane component, to an anhydrous bilayer, the lattice disordering effect of the spacer molecule is observed by spectral changes in C–H stretching and particularly the CH_2 deformation regions. Although chain–chain interactions are altered, no additional intrachain trans/gauche isomerization occurs on continued cholesterol intercalation. In general, the presence of cholesterol does not modify the structural rearrangements occurring on hydration alone. No evidence was found for cholesterol–lipid hydrogen bonding at the acyl chain carbonyl sites in either the anhydrous or completely hydrated bilayers.

The effects of adding one, two, and approximately four molecules of water per lipid molecule were observed by monitoring changes in vibrational frequencies and intensities characteristic of the head-group, interfacial, and acyl chain regions of the lipid bilayer matrix. There is no evidence that hydration leads to hydrogen bonds with the nonester PO_4^- oxygen atoms at low hydration levels. On the contrary, the spectral data indicate that hydration confers a mobility to the head-group, glycerol, and carbonyl moieties. A head-group conformational rearrangement, as indicated by simultaneous shifts in the C–N symmetric and PO_2^- antisymmetric stretching modes, occurs on the addition of approximately four molecules of water. Lattice disorder, reflected by the CH_2 deformation modes (1450-cm^{-1} region) and not the C–H stretching modes (2800–3100-cm^{-1} region), was induced by one water of hydration. Although a slight increase in trans/gauche isomerization occurs with two waters of hydration, significant intramolecular chain disordering arises with the addition of approximate four water molecules per lipid molecule. As noted above, these are the conditions favoring a head-group rearrangement.

The relevance of studying phospholipid bilayers ranging from a low water content to completely hydrated assemblies is appreciated through comparisons of the spectral data for DPPC assemblies containing either 10% (w/w) water or 70% (w/w) water. Except for a 1-cm^{-1} decrease in the C–N stretching mode, the spectral features of the 70% (w/w) gel system followed the same frequency and intensity patterns recorded in the 10% (w/w) bilayer spectra. Thus, after approximately four molecules of water are added to the system at a given temperature, no further head-group reorganization nor increases in either intrachain (trans/gauche) or lattice disorder take place on further hydration.

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Fetal Hemoglobin of the Rhesus Monkey, *Macaca mulatta*: Complete Primary Structure of the γ Chain†

Walter C. Mahoney and Peter E. Nute*

ABSTRACT: The complete amino acid sequence of the γ chain from the major one of two fetal hemoglobins from the rhesus monkey, *Macaca mulatta*, was determined by automated, stepwise degradation of selected fragments produced by cleavage at methionyl and tryptophanyl residues and at the single aspartylprolyl bond. The minor fetal hemoglobin is similar to human Hb F_1 in relative electrophoretic and chromatographic properties and in the level at which it is found (about 12% of the total Hb F). On these grounds, we assume that this minor component contains, like Hb F_1, γ chains that differ from those of the major component by virtue of acetylation of their amino-terminal glycyl residues. Although the γ chains of most anthropoid primates examined to date are structurally heterogeneous and, hence, appear to be encoded by nonallelic genes, no sign of structural heterogeneity was detected at any position in the major γ chain from *M. mulatta*. Thus, if nonallelic γ-chain genes exist in this species, the chains encoded by them may be identical in sequence. The γ chain from *M. mulatta* is but the sixth primate γ chain whose primary structure has been fully characterized. The slight extent of structural divergence among these chains (the four chains from various species of Old World monkeys differ from one another by no more than two substitutions, while the human and cercopithecoid γ chains differ at no more than five sites) attests to the conservative nature of γ-chain evolution among the higher primates.

Structural heterogeneity of γ chains in the fetal hemoglobins from single animals has served as a clue to the presence of nonallelic γ-chain loci in several species of anthropoid primates. The existence of nonallelic genes for $^{G}\gamma$ (with Gly in position 136) and $^{A}\gamma$ (with Ala in position 136) chains in normal human subjects was first illustrated through structural analysis of mixtures of the chains in question (Schroeder et al., 1968, 1972) and later demonstrated directly by gene mapping (Little et al., 1979; Fritsch et al., 1979; Tuan et al., 1979). In addition, there is compelling evidence, derived from structural analyses of the γ chains from gorillas (*Gorilla gorilla*) and chimpanzees (*Pan troglodytes*), that African apes also harbor nonallelic $^{G}\gamma$ and $^{A}\gamma$ genes (Huisman et al., 1973; De Jong, 1971). Nonallelic γ-chain genes may also exist in orangutans (*Pongo pygmaeus*), the γ chains of which are structurally heterogeneous at positions 75 (Ile/Val) and 135 (Ala/Thr) (Huisman et al., 1973; Schroeder et al., 1978). Among Old World monkeys (Cercopithecoidea), the fully sequenced γ chains of the baboon, *Papio cynocephalus*, are heterogeneous at position 75, where both valyl and isoleucyl residues appear [W. A. Schroeder, unpublished data cited in DeSimone et al. (1979); Nute & Mahoney, 1979a], while products of different γ-chain genes have been detected electrophoretically in lysates of red cells from each of 109 fetal and neonatal pig-tailed macaques (*Macaca nemestrina*) examined to date (Nute & Stamatoyannopoulos, 1971a,b; Nute & Mahoney, 1979b).

Of the primate species in which structurally distinct γ chains have been detected in all members examined, only *M. nemestrina* has two chains that are separable by standard procedures; hence, the majority of structural analyses of primate γ chains has been carried out on mixtures of no less than two different components. While there is no conclusive evidence that the γ chains from nonhuman primates other than those enumerated above are structurally heterogeneous, it must be emphasized that very few detailed analyses of primate fetal hemoglobins have been conducted. Given the presence of nonallelic γ-chain genes in man, as well as the likelihood that such exist in the great apes, pig-tailed macaques, and savannah baboons, it is reasonable to suspect that the γ chains of other higher primates are encoded by genes at more than one locus. In our continuing efforts to determine the extent of structural

† From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907 (W.C.M.), and the Departments of Anthropology (DH-05) and Medicine (Division of Medical Genetics) and the Regional Primate Research Center, University of Washington, Seattle, Washington 98195 (P.E.N.). *Received April 16, 1980.* This work was supported by Grants HL 20961, RR 00166, and EY 02038 from the National Institutes of Health, U.S. Public Health Service.

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