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EFFECT OF BILAYER CURVATURE ON VIBRATIONAL RAMAN SPECTROSCOPIC BEHAVIOR OF PHOSPHOLIPID-WATER ASSEMBLIES

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SUMMARY

In order to clarify the effect of bilayer curvature upon phospholipid conformation, vibrational Raman spectra were recorded for dipalmitoyl and dimyristroyl phosphatidylcholine in the gel state for both multilayer and single-wall vesicle assemblies. An intensity comparison, based upon a nonperturbing internal standard, between the two classes of bilayer systems reflected a decrease in peak height intensity for the observed hydrocarbon chain transitions in the single shell vesicle form. No intensity change between bilayer form was detected, however, for the two observed head group modes. Trends in the peak height intensity ratios for the 1100 cm^{-1} carbon-carbon stretching vibrations indicated an increase in hydrocarbon chain *trans-gauche* isomerization for the vesicle in comparison to the multilayer arrangements. The sensitivity of the methylene carbon-hydrogen stretching modes to interchain interactions was demonstrated by comparisons of the intensity patterns in the 2900 cm^{-1} region to the intensity characteristics of the carbon-carbon stretching region for polycrystalline, multilayer and vesicle materials. Examination of various carbon-carbon stretching mode intensity ratios for cholesterol doped dipalmitoyl phosphatidylcholine bilayers indicated that while 25 mol% cholesterol increased the *trans-gauche* acyl chain isomerization in multilayers, no comparable effect was observed for the vesicle forms. In contrast, the methylene twisting/methylene deformation intensity ratios for the cholesterol containing systems suggested that some further type of interchain perturbation occurs in the vesicle aggregations.

INTRODUCTION

Uniform, single-wall phospholipid vesicles are widely used as models for membrane studies involving a variety of structural and dynamical properties of bilayer aggregation (for examples, see refs. 1–3). The relevance of the unilamellar vesicle form stems from the compositional asymmetry between the inner and outer

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bilayer faces and the alterations in the packing arrangements of the phospholipid components, both of which are induced by the small radii of curvature of the single shell bilayer particle [2, 4–6]. Although the applicability of Raman spectroscopic techniques toward establishing structural characteristics of phospholipid molecules in natural and model membrane systems has been recently demonstrated [7–15], the effect upon phospholipid conformation of reducing multilamellar dispersions to single-wall vesicles by sonication methods has not clearly been established from vibrational analyses.

Recently, we compared the gel to liquid crystalline phase transition behavior of both multilayers and single-wall vesicles of dipalmitoyl and dimyristoyl phosphatidylcholine by monitoring the vibrational Raman spectroscopic frequency differences in the carbon-carbon (C-C) stretching region [16]. In particular, the phase transition for dipalmitoyl phosphatidylcholine was slightly lower in temperature and definitely broadened for the single shell vesicles as compared to the pure multilayer. This decrease in cooperativity associated with the phase transition suggested a more disordered state for the hydrocarbon chains of the vesicles. Although a clear distinction could not be made between the phase transitions for the multilayer and vesicle assemblies of dimyristoyl phosphatidylcholine, the intensities of transitions for modes other than the C-C stretching vibrations indicated structural differences in the vesicle forms for both the dimyristoyl and dipalmitoyl phospholipid molecules [16]. Mendelsohn et al. noted, however, that in their experiments sonication had no effect on the Raman 1132 cm^{-1}/1104 cm^{-1} C-C stretching intensity ratios for either egg phosphatidylcholine or dipalmitoyl phosphatidylcholine dispersions [17]. Thus, they concluded that no intramolecular structural disorder, in terms of changes in populations of the *trans* and *gauche* acyl chain isomers, occurred in the vesicle form [17]. Recent deuterium magnetic resonance studies indicated that the degree of order in both multilayers and vesicles is very similar [18]*. In the carbon-hydrogen (C-H) stretching region (2800–3000 cm^{-1}) of the Raman spectrum, sonication produced changes which Mendelsohn et al. [17] associated with interchain interactions.

In an effort to clarify further the effect of sonication on hydrocarbon conformations in lipid aggregations, we assess in the present study the relative intensity differences of specific vibrational Raman transitions of both dipalmitoyl and dimyristoyl phosphatidylcholine in their respective multi- and unilayer states. We also probe the dipalmitoyl phosphatidylcholine bilayer assemblies for the *trans-gauche* and interchain disorder which accompanies the addition of cholesterol to the lipid phase. All systems are examined below their gel to liquid crystalline phase transition temperatures.

* Calorimetric data are ambiguous as to the effects of sonication upon the thermodynamic properties of bilayer systems. For example, DeKruijff et al. [19] detected no significant changes in a comparison of the heat content of the phase transitions of dipalmitoyl and dimyristoyl phosphatidylcholine multilayers and vesicles. In contrast, calorimetric studies of Sturtevant [20] involving the vesicle forms of dipalmitoyl phosphatidylcholine definitely indicated a 5 °C decrease in the phase transition temperature and a 50 % enthalpy decrease, without loss in cooperativity, as compared to the behavior of multilayers.

EXPERIMENTAL

High purity samples of 1,2-dipalmitoyl-DL-phosphatidylcholine and cholesterol were obtained commercially from Sigma Chemical Co. Samples of L-α-1,2-dimyristoyl phosphatidylcholine were purchased from Calbiochem, Nutritional Biochemicals Corp. and Sigma Chemical Co. Since these substances produced only single spots by thin-layer chromatography methods and no spectral contaminants were observed, they were used without further purification. Dipalmitoyl phosphatidylcholine/25 mol% cholesterol mixtures were prepared by mixing previously weighed amounts of the substances in chloroform and drying first by a stream of nitrogen and then in vacuo. Multilayers were produced by agitating samples 30 % by weight of dipalmitoyl or dimyristoyl phosphatidylcholine or dipalmitoyl phsophatidylcholine/25 mol% cholesterol in water for 5 min with periodic warming to 45–50 °C. In some samples an aliquot of cacodylic acid [$(CH_3)_2As(O)OH$], 1.5 % by total weight, was added as an internal intensity standard. The cacodylic acid was always added after vortex mixing to insure that no water was lost through evaporation. These systems were mechanically mixed for 10 min with periodic heating to insure complete dispersal of the cacodylic acid.

Vesicles of approx. 250–300 Å in diameter [21] were prepared by sonicating samples 30 % by weight of dipalmitoyl or dimyristoyl phosphatidylcholine with water at 45–50 °C until optical clarity was reached. This was followed by centrifugation at 8000 rev./min at 0 °C for 30 min to remove larger vesicles and any remaining multilamellar material [5]. Resonication for 5–10 min after centrifugation was the final preparative step. A Branson sonifier, Model W-350, equipped with a microtip was used. 30 % by weight dipalmitoyl phosphatidylcholine vesicles incorporating 25 mol% cholesterol were also prepared using a bath type sonicator at about 45 °C. When cacodylic acid was used as an internal standard, its addition occurred after the vesicle preparation. The mixture was then gently agitated to disperse the reference material without incorporating it within the vesicles.

In order to verify for our experiments the preparation of homogeneous, small vesicle dispersions through sonication methods, we characterized the vesicle preparations by electron microscopy. The sonicated and centrifuged 30 % lipid samples were diluted to 0.1 % and equal volumes of the diluted sample and a solution of 2 % phosphotungstic acid for negative staining were mixed to give a final concentration of 0.05 % vesicles in 1 % phosphotungstic acid. This solution at pH 5.6 was subsequently dispersed onto a 200-mesh copper grid which had been previously coated with parlodion sprayed with a thin film of carbon and then deionized.

The grids with the applied vesicle preparations were observed on a Philips 201 electron microscope operating at 80 kV at a magnification level of 45 000. Calibration of the electron micrographs was by means of the crystalline spacings of suitably oriented crystals of indanthrene olive T dye [22]. The electron micrographs indicated the existence of single shell structures. Although areas of aggregated unilamellar vesicles were present, no severely deformed vesicles were visible.

Both polycrystalline and multilayer dipalmitoyl phosphatidylcholine Raman spectra were recorded from samples placed in capillary tubes. Other multilayer spectra were recorded from 1 cm diameter tubes. Vesicle spectra were obtained by multipassing the laser excitation beam through a 1 cm^2 cuvette and by collecting the scat-

tered light at 90° to the incident radiation. Sample temperatures, monitored by placing a copper-constantan thermocouple in the various solutions close to the laser beam transit, were maintained at 29±1 °C in a thermostated cell for dipalmitoyl phosphatidylcholine and at 14±2 °C for dimyristoyl phosphatidylcholine. Temperature control (±2 °C) for some samples of dimyristoyl phosphatidylcholine was maintained by directing a stream of cooled, dry nitrogen gas onto the sample receptacle. Adjustment of flow rate provided access to temperatures in the range −10 °C to room temperature. Spectra recorded at liquid nitrogen temperatures (−193 °C) were obtained with a cryostat which has been previously described [23].

Raman spectra were obtained with a Cary Model 81 spectrophotometer equipped with a modified external optical system and a Coherent Radiation Model 52 argon ion laser source. The laser was typically operated to give 300–900 mW of 5145 or 4880 Å radiation incident upon the sample. Spectral resolution varied between 2–5 cm^{-1}. Spectral frequencies calibrated with atomic argon lines, are reported to ±2 cm^{-1}.

RESULTS AND DISCUSSION

A. Pure dipalmitoyl and dimyristoyl phosphatidylcholine multilayer and single wall vesicle assemblies

Fig. 1 displays survey Raman spectra of polycrystalline, multilamellar and vesicular dipalmitoyl phosphatidylcholine in the 700–1500 cm^{-1} region. (These particular multilayer and vesicle spectra were recorded at 31±1 °C, while the polycrystalline spectra were recorded at 25±1 °C.) Although no shifts in the frequencies of the Raman transitions arise in a comparison of the three classes of dipalmitoyl phosphatidylcholine systems, the relative intensities of the contours in the 1300 and, in particular, the 1100 cm^{-1} regions of the multilayer and vesicle spectra change conspicuously in comparison to the spectrum of polycrystalline dipalmitoyl phosphatidylcholine. Fig. 2 details the 1065, 1100 and 1130 cm^{-1} transitions which are primarily related to C-C stretching motions [24, 25]. Both frequency differences and relative intensity changes for these vibrational modes as a function of temperature have been used by various investigators to monitor specific conformational changes in the hydrocarbon chains as bilayer lipid-water systems undergo gel to liquid crystalline phase transitions [12–17, 26]. The 1100 cm^{-1} region in particular has been shown to be a superposition of the C-C stretching modes for segments of the all-*trans* hydrocarbon conformations (in the gel or crystalline state of the system), the C-C modes of the hydrocarbon portions containing *gauche* bonds and the PO_2^- symmetric stretching mode [8–16]. An increase in intensity of the 1100 cm^{-1} band relative to the intensities of the 1065 and 1130 cm^{-1} transitions is indicative of a greater fluidity within the hydrocarbon chains; that is, an increase occurs in both the number and populations of various *gauche* rotamers. Since the spectra displayed in Fig. 2 were recorded at a constant sample temperature (in the gel state), the increase in the 1100 cm^{-1} band area relative to the 1065 and 1130 cm^{-1} band areas in the dipalmitoyl phosphatidylcholine multilayer and vesicle systems, respectively, as compared to polycrystalline dipalmitoyl phosphatidylcholine, originates from the increased intramolecular disorder in the multilayer and vesicle systems.

The differences in peak height intensities of individual transitions in the 1100

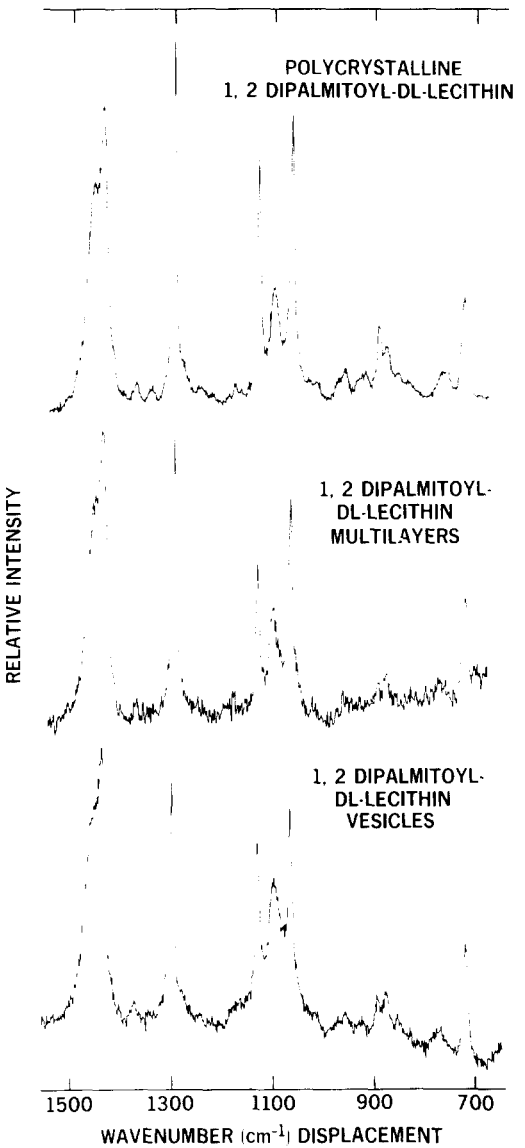

Fig. 1. Survey Raman spectra of the polycrystalline, multilayer and vesicle states of dipalmitoyl phosphatidylcholine in the 700–1500 cm^{-1} region utilizing 5145 Å excitation. The temperature of the polycrystalline material is 25 ± 1 °C, while the temperature for the multilayers and vesicles is 31 ± 1 °C.

cm^{-1} region as shown in Table I for multilayers and vesicles of both dipalmitoyl and dimyristoyl phosphatidylcholine, reflect the sensitivity of the Raman spectroscopic technique for monitoring conformational changes in bilayer systems. The multilayer and vesicle spectra from which the data were reduced were recorded at 29 ± 1 °C and 14 ± 2 °C for the dipalmitoyl and dimyristoyl systems, respectively. Temperatures were maintained at 10 °C below the phase transition temperatures to minimize fusion of the single-shell vesicles [27].

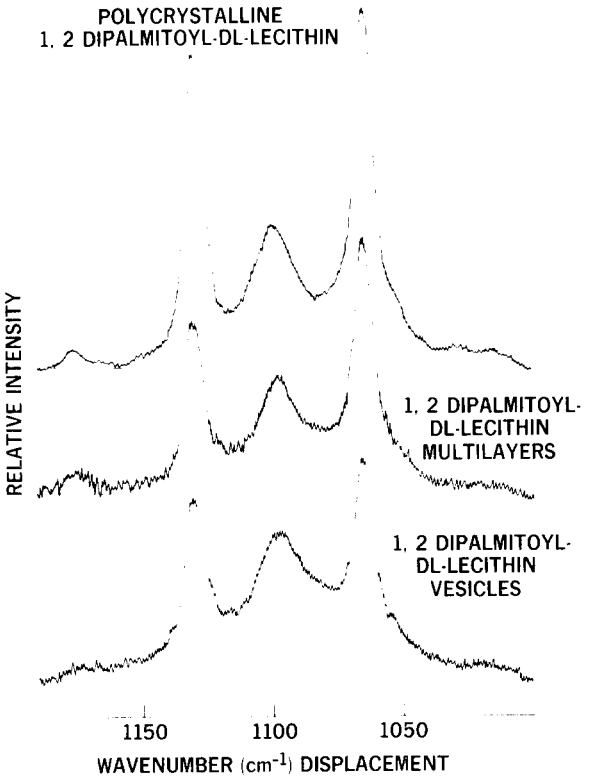

Fig. 2. Raman spectra of dipalmitoyl phosphatidylcholine in the polycrystalline, multilayer and vesicle forms in the 1020–1170 cm^{-1} region utilizing 5145 Å excitation. (See Fig. 1 legend for temperatures.)

A quantitative comparison was made between the multilayer and vesicle states by normalizing the observed peak heights to the 611 cm^{-1} transition of cacodylic acid (1.5 %)* which served as an internal standard. In comparing the intensities of multilayers and vesicles to an internal standard, we assume that the intrinsic scattering ability of the reference standard is not affected by the change in lamellar state of the phospholipid dispersion. In the absence of any spectral evidence that the reference compound binds or interacts with the lipid systems in excess water (70 %), we are confident that the scattering cross-sections of the internal standard are very nearly equivalent in the two classes of phospholipid dispersions. Since the phospholipids were present in concentrations of 30 % by weight in the multilayer and vesicle dispersions, quite reproducible Raman spectra with high signal to noise characteristics could be recorded. On the basis of comparisons with a number of spectra and

* This concentration of cacodylic acid caused no spectral perturbations to the vibrational transitions listed in the table. In addition, careful comparison between a variety of samples with and without this concentration of standard indicated that the reference compound produced no detectable conformational changes in the bilayer system. Concentrations of cacodylic acid from 0.5 % to several percent were added to the bilayer preparations with no apparent effect upon the *trans-gauche* ratios in the acyl chains. 1.5 % was chosen for use as the reference concentration for spectral convenience.

TABLE I

RELATIVE INTENSITY COMPARISON FOR DIPALMITOYL AND DIMYRISTOYL PHOSPHATIDYLCHOLINE MULTILAYERS AND VESICLES (1.5% CACODYLIC ACID USED AS AN INTERNAL STANDARD)

Peak heights in arbitrary units are no malized to the 611 cm^{-1} transition of cacodylic acid. Temperatures are 29 ± 1 °C and 14 ± 2 °C for dipalmitoyl and dimyristoyl phosphatidylcholine, respectively.

Frequency (cm^{-1})	Dipalmitoyl phosphatidylcholine			Dimyristoyl phosphatidylcholine			Assignments[b]
	Multilayers	Vesicles	Δ %[a]	Multilayers	Vesicles	Δ %[a]	
718	27	27	0	29	29	0	C-N stretch (choline)
767	5	5	0	5	5	0	O-P-O diester sym. stretch
1064	60	45.5	−24	55	45	−18	C-C stretch
1097[c]	27	28	+ 4	29	27.5	− 5	C-C stretch + PO_2^- sym. stretch
1129	44.5	39	−12	39	29.5	−24	C-C stretch
1297	77.5	53.5	−31	67.5	50	−26	CH_2 twist
1438	77.5	59	−24	77	67	−13	CH_2 deformation
1455	58	44	−24	60	55	− 8	
2847	248.5	250	0	218	181	−17	CH_2 sym. stretch
2882	319	268	−16	286	209	−27	CH_2 asym. stretch

[a] $\Delta = (V - M)/M \times 100$, where V and M represent the peak height intensities of the vesicles and multilayer transitions, respectively.

[b] Ref. 8; sym. represents symmetric and asym. asymmetric.

[c] This transition occurs at 1090 cm^{-1} in dimyristoyl phosphatidylcholine bilayer systems.

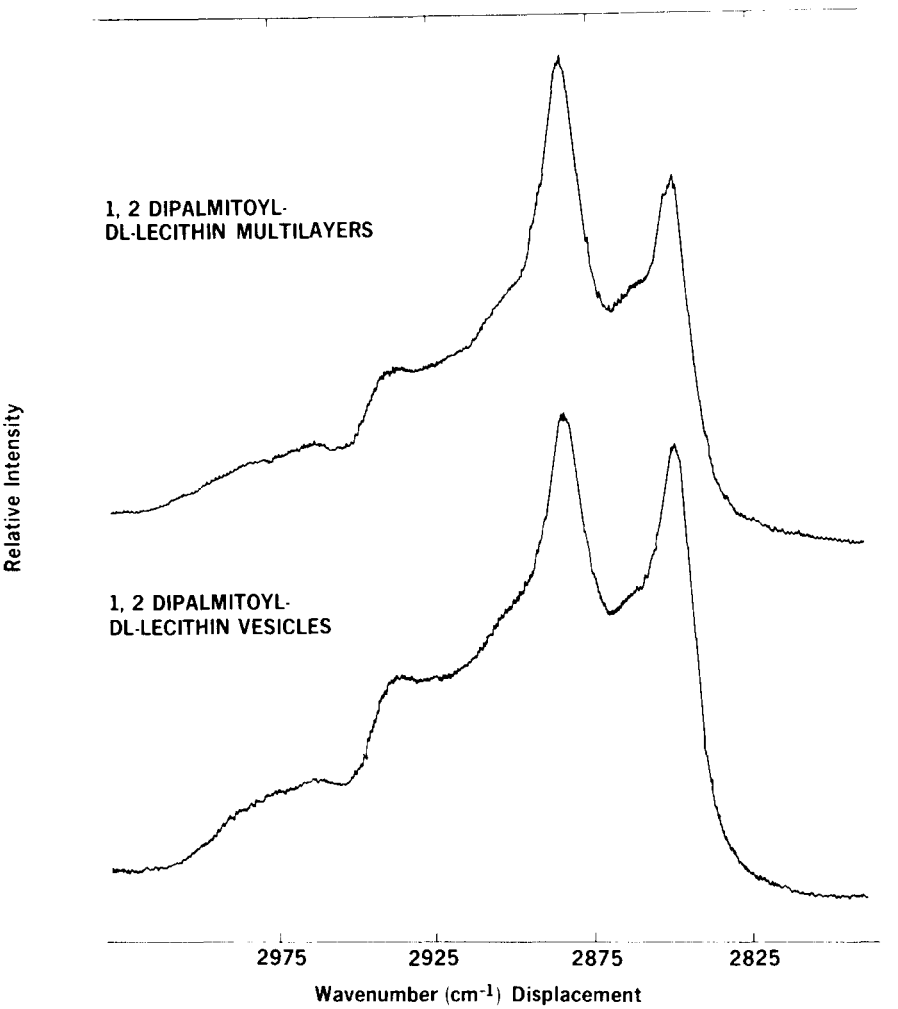

Fig. 3. Raman spectra of dipalmitoyl phosphatidylcholine multilayers and vesicles at 29 °C in the 2800–3000 cm^{-1} region utilizing 5145 Å excitation.

samples we estimate the maximum variability in the measured peak heights recorded in Table I to be approximately 5 %. Limited data were obtained with vesicle dispersions of 10–20 % by weight of phospholipid. These data appeared entirely consistent with that of the more concentrated vesicle dispersions.

Fig. 3, which displays the C-H stretching region for both dipalmitoyl phosphatidylcholine multilayer and vesicle systems recorded at 29 ± 1 °C, provides additional Raman spectroscopic evidence for structural dissimilarities in the hydrocarbon chains of these systems. The 2882 cm^{-1} CH_2 asymmetric stretching mode in the vesicle spectrum has a significantly lower intensity relative to the 2847 cm^{-1} symmetric CH_2 stretching mode as compared to the multilayer spectrum. This ob-

servation was noted by Mendelsohn et al. for the dipalmitoyl bilayer system [17]*. Table I also summarizes the methylene stretching intensities observed for both dipalmitoyl and dimyristoyl phosphatidylcholines multilayer and vesicle systems, respectively.

The CH_2 twisting mode at 1297 cm^{-1} (Fig. 1) is a further Raman transition which has been shown to be conformationally dependent in temperature studies of dipalmitoyl phosphatidylcholine multilayers [9, 16, 17, 26]. We note the rather dramatic decrease in its intensity relative to the 1438 cm^{-1} CH_2 deformation mode in going from the polycrystalline to a vesicle conformation. Further, as shown in Table I, a more quantitative comparison reveals a substantial peak height intensity decrease of this feature in the vesicle forms as compared to the multilayer assemblies of the model systems. Since the 1297 cm^{-1} transition undergoes changes in line shape and intensity as bilayer systems undergo gel to liquid crystalline transition [9, 16, 17, 26], intensity alterations in this transition between multilayers and sonicated material also imply some combination of intramolecular and interchain disorder.

In general, changes in Raman intensities could be considered to originate primarily from several sources; namely, the field effects which are related to the dielectric constant (or refractive index) of the medium surrounding the scattering center, changes in the normal coordinates specifying the vibrations and chemical effects, such as electron-donor-acceptor interactions, that perturb specifically the charge distributions about the vibrating nuclei. For assessing intensity changes between multilayer and vesicle states, the first two effects are probably predominant. Since the dielectric constant (and therefore the refractive index) is postulated to be greater for a vesicle system [28], the latter phospholipid form would be expected to exhibit an enhanced Raman intensity [29, 30]. The overall decrease in intensity for vesicles (Table I) indicates that the normal coordinate changes for the hydrocarbon vibrations are significant to the extent of reversing the field effect difference in going to the vesicle state characterized by small radii of curvature. Although our instrumental resolution is insufficient for determining at a given temperature small, absolute frequency shifts, which would relate to normal coordinate differences between the two bilayer forms, significant frequency and intensity variations are observed in temperature studies where, for example, the C-C stretching normal coordinates vary [16]. Thus, we conclude that the intensity decrease, for the hydrocarbon transitions in the minimum radius vesicles, indicates a change in the acyl chain structure (both inter- and intrachain disorder) which in turn affects the vibrational normal coordinates.

In their recent Raman study on dipalmitoyl phosphatidylcholine, Mendelsohn et al. [17] concluded that sonication induced either little or no changes in the amounts of hydrocarbon *trans* and *gauche* isomers present at specified temperatures below and above the phase transition. The authors also concluded on the basis of intensity changes in the C-C stretching region that the all-*trans* conformation predominates at room temperature. In the present study we limit the discussion of the effects of sonication upon conformational changes to only the gel state below the phase transition.

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* The altered 2900 (and 1100) cm^{-1} band patterns for dipalmitoyl phosphatidylcholine vesicles at 29 °C closely resemble those produced when the polycrystalline material is heated to about 60 °C. (Spiker, Jr., R. C. and Levin, I. W., unpublished data). This is still not the point at which complete disorder is observed, as is the case, for example, when dipalmitoyl phosphatidylcholine is dissolved in chloroform.

TABLE II

COMPARISON OF PEAK HEIGHT INTENSITY RATIOS OF (A) CARBON-CARBON STRETCHING AND (B) CARBON-HYDROGEN STRETCHING TRANSITIONS FOR DIPALMITOYL AND DIMYRISTOYL PHOSPHATIDYLCHOLINE MULTILAYER AND VESICLE FORMS

A. Bilayer system	Temperature (°C)	I (1129 cm^{-1})/ I (1097 cm^{-1})[a]	I (1064 cm^{-1})/ I (1097 cm^{-1})	I (1129 cm^{-1})/ I (1064 cm^{-1})
Dipalmitoyl phosphatidylcholine vesicles[b]	−193	3.35	2.97	1.13
Dipalmitoyl phosphatidylcholine (anhydrous)	25	2.16	2.47	0.88
Dipalmitoyl phosphatidylcholine multilayers	29	1.65	2.22	0.74
Dipalmitoyl phosphatidylcholine vesicles	29	1.39	1.63	0.86
Dimyristoyl phosphatidylcholine vesicles[c]	−193	2.89	2.83	1.02
Dimyristoyl phosphatidylcholine multilayers[d]	14	1.34	1.90	0.71
Dimyristoyl phosphatidylcholine vesicles	14	1.07	1.63	0.66
B.		I (2882 cm^{-1})/ I (2847 cm^{-1})		
Dipalmitoyl phosphatidylcholine (anhydrous)	25	1.32		
Dipalmitoyl phosphatidylcholine multilayers	29	1.28		
Dimyristoyl phosphatidycholine multilayers	14	1.31		
Dipalmitoyl phosphatidylcholine vesicles	29	1.07		
Dimyristoyl phosphatidylcholine vesicles	14	1.15		

[a] Transitions in heading refer to dipalmitoyl phosphatidylcholine at 29 °C

[b] Transitions occur at 1134, 1103 and 1065 cm^{-1} at liquid nitrogen temperatures. Low temperature samples were annealed to insure an all-*trans* hydrocarbon conformation at these temperatures.

[c] Transitions occur at 1132, 1094 and 1064 cm^{-1} for dimyristoyl phosphatidylcholine bilayers at liquid nitrogen temperatures.

[d] Transitions occur at 1129, 1090 and 1064 cm^{-1} for dimyristoyl phosphatidylcholine bilayers at 14±2 °C.

Mendelsohn et al. based their conclusions solely upon comparisons of the intensity ratios between the 1129 and 1097 cm^{-1} transitions [17]. Since normal coordinate studies on a variety of hydrocarbon conformations [24, 25] associate the three transitions in the 1050–1150 cm^{-1} region with C-C stretching modes, a comparison of the changes in intensity throughout this specific spectral area (as suggested by Table I) may better indicate relatively subtle variations in the number of *trans* and *gauche* conformers from system to system. Table II, then, summarizes the relative intensity ratios for the three primary transitions in the C-C stretching region. The relative intensities of these transitions for dipalmitoyl phosphatidylcholine change rather dramatically between liquid nitrogen temperatures (−193 °C) and 29 °C, indicating that significant alterations to the all-*trans* conformation (assumed to be present in the annealed sample at −193 °C) occur for an increase in temperature. In addition to the usual spectral changes associated with the growth of *gauche* chain structures, the intensities of the two outer C-C stretching transitions (1129 and 1064 cm^{-1}) have reversed (compare rows 1 and 2, Table II, for I(1129 cm^{-1})/I(1064 cm^{-1})). In addition, a 5 cm^{-1} decrease in frequency arises for the 1129 cm^{-1} transition as the temperature changes from −193 to 29 °C. Except for the I (1064 cm^{-1})/I (1097 cm^{-1}) ratio for dipalmitoyl phosphatidylcholine, which is clearly a function of bilayer form, the comparisons of the remaining ratios involving the *gauche* 1097 cm^{-1} band are either close to or somewhat within the 10 % range of maximum experimental error in the measured quantity for each bilayer system. We do note, however, that for the specific temperatures defining the experiment, the values for the intensity ratios involving the 1097 cm^{-1} transition for dipalmitoyl phosphatidylcholine and the 1090 cm^{-1} transition for dimyristoyl phosphatidylcholine are unmistakably in the direction expected for an increase in *gauche* conformers as the systems change from the multilayer to vesicle form. This trend is consistently reproduced in the spectra of new multilayer and vesicle phospholipid samples. In summary at this point, the relative intensity ratios for the C-C stretching modes strongly suggest a trend toward increased disorder in the vesicle state, although the detection of differences in structure is approaching the limits in sensitivity of the Raman spectroscopic technique. The actual peak height intensities, measured against an internal standard, are, however, definitely decreased for the vesicle hydrocarbon transition.

The frequency dependence of the 1129 cm^{-1} feature for the dipalmitoyl and dimyristoyl C-C stretching mode has been discussed in detail previously [16]. We add the observation here that the intensity and frequency behavior of this transition in the dimyristoyl system is more sensitive to temperature changes and apparent packing rearrangements than in the dipalmitoyl system. In addition, the methylene CH stretching modes (2882 and 2847 cm^{-1}) are significantly less intense in the dimyristoyl multilayer and vesicle systems as compared to the dipalmitoyl counterparts. This absolute decrease in intensity may be relevant either to the rippled lamellae that dimyristoyl phosphatidylcholine forms in the Pβ' phase [31], or to a structural change in which the tilted hydrocarbon chains become oriented perpendicularly to the bilayer plane [32]. Since the temperature for the dimyristoyl system lies in the pretransition region, an analogy to the structural changes occurring in the pretransition region for the dipalmitoyl system [32] may be appropriate. (It should also be noted that the Pβ' phase for dimyristoyl multilayers was only studied in systems with low water content [32] in contrast to our systems). The ratios of I (2882 cm^{-1})/I (2847 cm^{-1}),

recorded in Table II, part B, remain nearly constant for the multilayer systems of both dipalmitoyl and dimyristoyl phosphatidylcholine. The same observation is made for the vesicle forms for both lipid types.

Since the intensity changes in the CH_2 stretching transitions reflect to some extent the *trans-gauche* conformational modifications which occur during the melting of hydrocarbon chains, these spectral features were used to monitor the gel to liquid crystalline transition of lipid bilayers [9]. Mendelsohn et al. [17, 26] suggested that the methylene stretching modes also reflect interchain interactions, as a consequence of their relatively large vibrational amplitudes, and are therefore sensitive to perturbations in packing rearrangements. These ideas are supported by the equivalence of the I (2882 cm^{-1})/I (2847 cm^{-1}) ratios for anhydrous dipalmitoyl phosphatidylcholine (at 25° C) and the dipalmitoyl multilayer system (at 29 °C) in conjunction with the inequivalence of the I (1128 cm^{-1})/I (1097 cm^{-1}) ratios (which are sensitive to *trans-gauche* isomerizations) for the two systems, respectively (see Table II). A perhaps related point is the particularly unique sensitivity of C-H stretching modes to environmental effects which has been observed in absolute infrared intensity measurements of binary solution mixtures. This phenomena has tentatively been attributed to a perturbation of the hydrogen stretching normal modes through primarily the repulsive forces present in the solute-solvent interaction [33].

In the present discussion we have referred only to the structure sensitive transitions associated with the hydrocarbon portions of the phospholipid molecules. Unfortunately, we are limited to examining only two of the head group vibrations in spectroscopically monitoring the multilayer and vesicle systems by Raman techniques. These vibrations are the 718 cm^{-1} C-N choline symmetric stretching and the 767 cm^{-1} phosphate diester symmetric stretching modes [8]. We observed no differences in Raman intensities of these vibrations between the multilayer and vesicle forms of either the dipalmitoyl or dimyristoyl phosphatidylcholine molecules. The intrinsic weakness of the 767 cm^{-1} feature, however, makes intensity comparisons difficult for small changes. Since the 718 cm^{-1} feature, which is moderately intense, remains constant in intensity between multilayers and vesicle systems, this transition may also serve as internal standard. The failure to find a conformationally dependent head group Raman vibration is consistent with ^{31}P NMR studies in which it was determined that sonication did not significantly alter the structure of the phosphate region [34].

B. Cholesterol doped multilayer and vesicle states

Although it is clear from Raman spectra [13, 16], as well as from calorimetric data [35, 36], that the addition of cholesterol broadens the phospholipid bilayer gel to liquid crystalline phase transition, it is of interest to examine the effect of cholesterol upon the packing and disorder characteristics present in single-wall vesicles. Table III summarizes the Raman spectral comparisons for multilayer and sonicated vesicle systems of dipalmitoyl phosphatidylcholine with the addition of 25 mol% cholesterol at 29±1 °C. Based upon the 611 and 707 cm^{-1} cholesterol reference peaks, contributions to the hydrocarbon spectra from cholesterol were not important for this mole fraction of cholesterol except in the 2800–3000 cm^{-1} methylene stretching region; consequently, no data are reported for this spectral interval. The I (1129 cm^{-1})/I (1097 cm^{-1}) intensity ratio for the multilayers shows particularly the in-

TABLE III

COMPARISON OF RELATIVE INTENSITIES OF SELECTED RAMAN TRANSITIONS FOR DIPALMITOYL PHOSPHATIDYLCHOLINE MULTILAYERS AND VESICLES CONTAINING 25 MOL PERCENT CHOLESTEROL

Intensity[a] ratio	Polycrystalline dipalmitoyl phosphatidylcholine	Dipalmitoyl phosphatidylcholine			
		Multilayers		Vesicles	
		No cholesterol	25 Mol % cholesterol	No cholesterol	25 Mol % cholesterol
I (1064 cm^{-1})/I (1097 cm^{-1})[b]	2.47	2.22	2.22	1.63	1.72
I (1129 cm^{-1})/I (1097 cm^{-1})	2.16	1.65	1.39	1.39	1.25
I (1297 cm^{-1})/I (1440 cm^{-1})	1.18	1.00	0.83	0.91	0.73

[a] Temperature of polycrystalline system was 25 C. Temperature of bilayers was 29 ± 1 °C.

[b] The 1097 cm^{-1} Raman transition shifts to 1095 cm^{-1} when cholesterol is incorporated into multilayer and vesicle assemblies.

crease in *gauche* structure due to the addition of cholesterol, with the degree of disorder at this temperature approximating that of the vesicle state. The I (1064 cm^{-1})/I (1097 cm^{-1}) ratio does not reflect a change on addition of cholesterol. Using both intensity ratios as a probe of chain disorder, we note that the addition of cholesterol to vesicles is not reflected by an increase in *gauche* conformers. In contrast, however, the I (1297 cm^{-1})/I (1440 cm^{-1}) ratios, in which 1297 and 1440 cm^{-1} are the CH_2 twisting and deformation modes, respectively, exhibit distinct changes as cholesterol is added to multilayer and vesicle assemblies. Since the 1450 cm^{-1} region of crystalline polyethylenes is particularly sensitive to interchain interactions [37], changes in the I (1297 cm^{-1})/I (1440 cm^{-1}) intensity ratios probably reflect variations in packing arrangements of the hydrocarbon chains for cholesterol containing systems. These results are consistent with the studies of Gent and Prestegard [38] in which the radii of egg yolk phosphatidlycholine single shell vesicles increase with increasing cholesterol composition. That is, variations in the radii of dipalmitoyl phosphatidylcholine vesicles which might arise from the addition of cholesterol would probably be reflected by the packing characteristics of the hydrocarbon chains.

CONCLUSION

Although the Raman spectra of sonicated single-wall vesicles reflect a distinctly broadened gel to liquid crystalline phase transition in contrast to the sharp behavior of multilayers, ambiguities remain with respect to the details of disorder that are individually characteristic of a bilayer type [16, 17]. In particular, it was deduced from recent deuterium nuclear magnetic resonance (^2H NMR) studies [18] that the degree of order is very similar in both types of bilayers with the differences arising primarily in the portions of the hydrocarbon chains near the bilayer center. For these specific acyl segments the ^2H NMR results indicated a decrease in a defined order parameter of 10–30 % for the vesicle systems in comparison to the multilayers. In the present Raman study we assess the sensitivity and utility of vibrational spectra toward distinguishing structural differences between multilamellar liposomes and minimum radii vesicles. Sonicated preparations were examined by electron microscopy to insure the existence of single-shell vesicle forms. Raman spectra for dipalmitoyl and dimyristoyl phosphatidylcholine multilayers and vesicles were compared at 10 °C below the phase transition in order to minimize the possibility of fusion within the vesicles.

Intensity differences in both bilayer states were compared through reference to an internal standard. The overall decrease in intensity of the hydrocarbon transitions for vesicles indicates that significant changes in packing characteristics are induced in systems with small radii of curvature. Although the limit in sensitivity of the Raman technique is approached, definite trends in the peak height intensity ratios for I (1129 cm^{-1})/I (1097 cm^{-1}) and I (1064 cm^{-1})/I (1097 cm^{-1}) C-C stretching vibrations indicate increased intrachain disorder, or greater *trans-gauche* isomerization, for the vesicle state in comparison to the multilayer arrangements. Unfortunately, in the absence of deuterated hydrocarbon chains, the Raman technique cannot locate the specific segments exhibiting *gauche* behavior. The sensitivity of the CH_2 stretching modes to interchain disorder is demonstrated by comparing the patterns for the I (2882 cm^{-1})/I (2847 cm^{-1}) intensity ratios for polycrystalline, multilayer

and vesicle materials to the intensity patterns characteristic of only the C-C stretching modes. Specifically, the CH_2 stretching intensity ratios reflect the same general packing environment for the polycrystalline and multilayer states in the dipalmitoyl system, while the concomitant C-C stretching intensity ratios indicate greater intrachain disorder for the multilayer material (perhaps toward the center of the bilayer) in comparison to the anhydrous sample. It is difficult to interpret the further change in CH_2 intensity ratios between the multilayer and vesicle forms in terms of relative contributions of either inter- or intrachain disorder. Temperature dependent spectral studies, which are now in progress, should prove helpful in attributing these two effects to specific intensity changes or frequency shifts in the vibrational data (Yellin, N. and Levin, I. W., unpublished observations).

Only two vibrational modes associated with the polar head group could be monitored for structural changes involving bilayer curvature. Neither transition, the C-N symmetric stretching mode nor the phosphate diester symmetric stretching vibration, shows an intensity alteration between the multilayer and vesicle states. This observation suggests that bilayer curvature fails to influence the head group conformation.

Examination of various intensity ratios for dipalmitoyl phosphatidylcholine/25 mol% cholesterol/water mixtures indicates that while 25 mol% cholesterol increases the population of *trans-gauche* acyl isomers in multilayers, no effect is observed for vesicle systems. That is, the intrachain disorder induced by the small radii of curvature of the vesicles is not further increased by this concentration of cholesterol. In contrast, the CH_2 twisting/CH_2 deformation intensity ratios, which reflect interchain effects, indicate that the intercalated cholesterol in vesicles continues to perturb the phospholipid packing constraints, probably by expanding slightly the vesicle size [38]. Since small radius vesicles formed from phosphatidylcholine/cholesterol mixtures preferentially distribute cholesterol within the inner face of the bilayer [2], Raman spectra may provide an additional physical means for selectively probing the inner surface of the vesicle wall.

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