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A Raman spectroscopic study on the effect of cholesterol on lipid packing in diether phosphatidylcholine bilayer dispersions

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For assessing lipid-sterol packing characteristics in model membrane systems, the vibrational Raman spectra of 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (DHPC) multilamellar dispersions containing 18 mol% cholesterol were examined. The thermotropic behavior of the pure diether- and cholesterol-containing bilayers were studied in both the C-H stretching (2800–3100 cm^{-1}) and C-C stretching (1000–1200 cm^{-1}) mode regions, spectral intervals reflecting intermolecular and intramolecular order/disorder characteristics. Pure DHPC bilayers exhibit the pretransition and gel to liquid-crystalline phase transition temperatures at approx. 31 and 42.8°C, respectively. In contrast to the temperature behavior of dipalmitoylphosphatidylcholine-cholesterol bilayers in which the primary phase transition T_m, is simply broadened, T_m is increased to approx. 49.6°C in the DHPC (diether PC) liposomes containing 18 mol% cholesterol. As in the saturated chain diacyl-cholesterol bilayer systems, the sterol disorders the DHPC gel phase and eliminates the pretransition, while ordering the liquid-crystalline phase. Pure diether liposomes exhibit a headgroup dehydration effect as the multilamellar dispersions are cycled slowly over a temperature range including the gel and liquid-crystalline forms. That is, on multiple passages through the phase transitions, both the gel and liquid-crystalline bilayer forms assume an increased hydrocarbon chain order. In the cholesterol-containing diether bilayers, only the disordered intra- and interchain states are formed.

Introduction

Because of both the predominance and importance of cholesterol in cellular membranes, numerous studies encompassing a variety of physical techniques have attempted to clarify the underlying cholesterol-lipid interactions defining bilayer reorganizations as a function of changes in either sterol concentration or membrane temperature (Refs. 1–3 and references contained therein). Recognition that membrane structural and functional properties may be altered by substituting ether linkages for the lipid diester bonds within the bilayer matrix has led to the use of comparative studies between diester and diether lipid dispersions as a means for discerning the salient structural features relating lipid-lipid, lipid-sterol and lipid-protein interactions to membrane behavior [4–6]. For examining alterations in membrane structure provoked by either cholesterol or other bilayer-associated species, vibrational Raman spectroscopy particularly provides a sensitive monitor of the changes of molecular order within the bilayer matrix. From the spectral frequency and intensity data recorded specifically in the hydrocarbon chain carbon-hydrogen and carbon-

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Abbreviations: DHPC, 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; DPPC, dipalmitoylphosphatidylcholine.

carbon stretching mode intervals, bilayer order/disorder characteristics within the hydrophobic region may be interpreted in terms of changes in the lateral interactions between chains and in redistributions of the relative populations of *trans* and *gauche* conformers along the chain [7–9].

In the present communication we apply Raman spectroscopic techniques to describe first the gel and liquid-crystalline polymorphism exhibited by diether lipid multilamellar aqueous dispersions formed from DHPC. These specific structural reorganizations do not have direct counterparts in dispersions comprised of diacyl lipids. We then investigate the effect of cholesterol on the diether lipid bilayer in terms of changes in the intra- and intermolecular order/disorder parameters derived from Raman spectra. Since phase boundaries have been suggested for acyl lipid bilayer systems containing 20, 33 or 50 mol% cholesterol [1], we restrict this study to DHPC bilayers containing 18 mol% cholesterol; that is, a cholesterol-containing system which allows sufficient sterol-lipid complexation to occur in order to be observed spectroscopically, but at a sterol concentration below which additional complicating membrane effects may arise.

Experimental Procedures

Polycrystalline samples of DHPC and cholesterol were commercially obtained from the Calbiochem-Behring Corp. and the Sigma Chemical Company, respectively. Since no contaminants were observed in the vibrational spectra in either polycrystalline system, both materials were used without further purification.

Lipid dispersions were prepared by mechanically agitating a 3 : 1 (w/w) mixture of water and DHPC. The aqueous dispersion was heated to approx. 50°C and mechanically agitated to insure complete sample hydration. The dispersion was then transferred to a capillary sample tube, centrifuged, covered with excess water and sealed. Each tube was sealed and incubated from 3 to 7 days at −14°C. For the preparation of cholesterol-containing liposomes at concentrations of 18 mol%, appropriate amounts of DHPC were added to chloroform (spectral grade) solutions of cholesterol. These mixtures were first vigorously agitated; chloroform was then removed by initial passing a stream of nitrogen over the samples to evaporate bulk chloroform. The samples were then placed under vacuum for 18–24 h at approx. 10^{-4} Torr for further drying. Hydrated dispersions for the Raman experiments were prepared as described above.

Raman spectra were recorded at a spectral resolution of 3–4 cm^{-1} using a Spex Ramalog 6 spectrometer equipped with holographic gratings and interfaced to a Nicolet NIC-1180 data acquisition system. A Coherent Model CR-12 argon ion laser provided at the sample approx. 200 mW of excitation power at 514.5 nm. The frequencies, calibrated with atomic argon lines, are reported to ± 2 cm^{-1}. Spectra reflect 4 or 5 scans for the C-H stretching mode region (3000 cm^{-1}) and 8–10 scans for the C-C stretching mode regions (1000 cm^{-1}); scan rates of 1 cm^{-1}/s were utilized for data collection. Spectra were also recorded on a Spex 1877 Triplemate spectrograph using an EG and G Model 1420 intensified silicon photodiode array detector. Data were acquired by a local LSI-11 based computer with a Tektronix 4006 graphics terminal for communicating with a laboratory PDP 11-70 computer for data storage and manipulation. 15–30 mW of power were supplied to the sample by a Spectra Physics Model 165-07 laser. Temperature-dependent spectra were generated with both instruments in an ascending mode with equilibration times of 12.5 min between consecutive points.

Temperature profiles for the multilamellar assemblies were constructed from the C-H and C-C stretching mode spectra using the Raman peak height intensity ratios, $I(2846\ cm^{-1})/I(2882\ cm^{-1})$ and $I(2934\ cm^{-1})/I(2882\ cm^{-1})$, to represent interchain disorder/order parameters. The $I(1090\ cm^{-1})/I(1130\ cm^{-1})$ intensity ratios provide markers for identifying changes in the intrachain *gauche/trans* rotational isomer populations [10]. Neither spectra deconvolution nor smoothing procedures were applied. Spectral contributions to the lipid C-H and C-C stretching mode regions originating from the cholesterol sample were computer subtracted before constructing the temperature profiles for the cholesterol-containing liposomes. Spectra of crystalline cholesterol were used as the reference spectra in the subtraction steps.

Results and Discussion

Fig. 1 presents the 2800–3100 cm^{-1} C-H stretching region spectra of the low-temperature gel state and high-temperature liquid-crystalline phase of DHPC bilayers for two polymorphic forms. The C-H stretching region spectra generally reflect lateral chain-chain interactions, but may also be sensitive to a superposition of spectral effects originating from intrachain conformational disordering [11,12]. Since the vibrational assignments of this complex region have been discussed in detail previously [7,10], we only note here that intensity and frequency changes in the 2846, 2882 and 2934 cm^{-1} features observed for the gel state diether lipid provide useful markers for assessing bilayer rearrangements in the hydrophobic region of the assembly. These three spectral transitions are assigned, respectively, to the hydrocarbon chain methylene C-H symmetric stretching modes, the methylene C-H asymmetric modes and, in part, a Fermi resonance component of the chain terminal methyl C-H symmetric stretching mode.

The spectral patterns obtained after lipid hydration, rapid cycling between the gel and liquid crystalline phases and then annealing at −14°C for 3–7 days are shown in Figs. 1A and B. After obtaining several temperature profiles on a single lipid dispersion, as described above, the new spectral patterns that arise are shown in Fig. 1C and D. Thus, although a sample may have been incubated at −14°C for several days between temperature scans, the more often a temperature profile was obtained on a given sample, the more likely were the gel and liquid-crystalline spectra to exhibit patterns approaching those of Fig. 1C and D. Since the relative peak height intensity ratios of the 2850, 2880 and 2935 cm^{-1} features reflect lattice expansion effects, as well as *trans/gauche* isomerization changes [10], we conclude that the spectrum in Fig. 1D represents a more ordered liquid-crystalline state than that shown in Fig. 1C [10,13]. For the cholesterol-containing bilayers, only the spectrum shown in Fig. 1B, the more disordered liquid-crystalline spectrum, was observed, even after completing multiple temperature profiles with a given sample. This behavior of both pure and cholesterol-containing DPHC bilayers may be understood by first recognizing that lipid-cholesterol interactions are accompanied by an increase in the amount of bound water in the headgroup [14]. Vibrational Raman spectroscopic studies have demonstrated that as anhydrous bilayer samples of dipalmitoylphosphatidylcholine (DPPC) bind water (with one, two and approximately four molecules of water per lipid molecule),

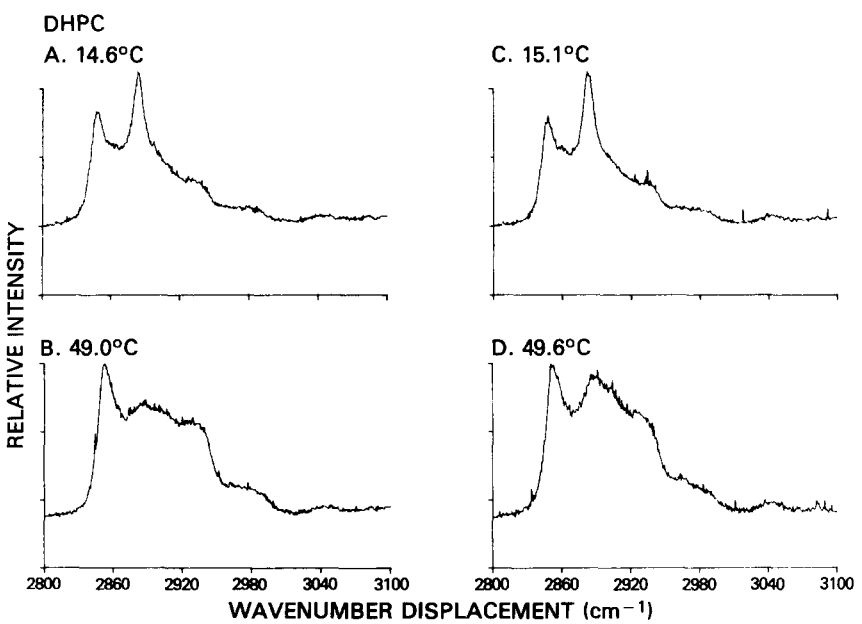

Fig. 1. Raman spectra of aqueous dispersions of DHPC in the 2800–3100 cm^{-1} C-H stretching mode region. (A) and (B) represent the gel and liquid crystalline forms, respectively, for dispersions whose thermal history involved only lipid hydration and incubation at −4°C for 3–7 days prior to obtaining temperature profiles. (C) and (D) represent the more ordered gel and liquid crystalline forms, respectively, of dispersions for which multiple temperature scans were obtained in both the gel and liquid-crystalline phases.

the acyl chains show a definite trend toward lattice disorder [15]. Although the present pure and cholesterol containing DHPC bilayers do not exhibit these effects as profoundly in the gel state for the C-H stretching region spectra, the liquid-crystalline spectra (Fig. 1B) are clearly consistent with a greater disordering of the hydrocarbon chains as might occur for an increase in the amount of bound water within the headgroup. Further evidence demonstrating the effect of bound water on chain disorder stems from examining the temperature behavior of anhydrous, polycrystalline DHPC. In particular, melted bilayers of anhydrous DHPC yield spectral patterns analogous to Fig. 1D (Harris and Levin, unpublished data); that is, the more ordered bilayer form. We conclude that as the pure DHPC bilayers are slowly cycled through their temperature profiles, the headgroups undergo a dehydration effect. The ability of the diether chains to pack more closely in comparison to the diacyl lipids may compete with the tendency for hydration spheres of bound water to occur within the polar headgroup region. Thus, as the van der Waals interactions between chains lead to a denser bilayer matrix packing, the interface headgroup lipid regions would become restricted in directly binding water.

The bilayer lattice ordering effect due to the dehydration of a headgroup is observed to a lesser degree in the gel state of the DHPC assembly. In general, the peak height intensity ratio $I(2850\ cm^{-1})/I(2880\ cm^{-1})$ yields a measure of the extent of the lateral chain-chain interactions within the lipid bilayer, as well as providing an indication of the subcell arrangement [16]. Qualitatively, an increase of the 2882 cm^{-1} feature relative to the 2840 cm^{-1} transition between bilayer systems implies increased lateral interactions. The decrease in the $I(2850\ cm^{-1})/I(2880\ cm^{-1})$ ratio from 0.74 for the initial DHPC gel state bilayer (Fig. 1A) to 0.69 (Fig. 1C) for the gel state attained after multiple cycling through the phase transition confirms the more ordered lattice expected to result from headgroup dehydration.

Recent *X*-ray diffraction studies indicate that DHPC gel-state bilayers are interdigitated [17]. Although the $I(2850\ cm^{-1})/I(2880\ cm^{-1})$ ratio of 0.69 for the spectrum shown in Fig. 1C is con-

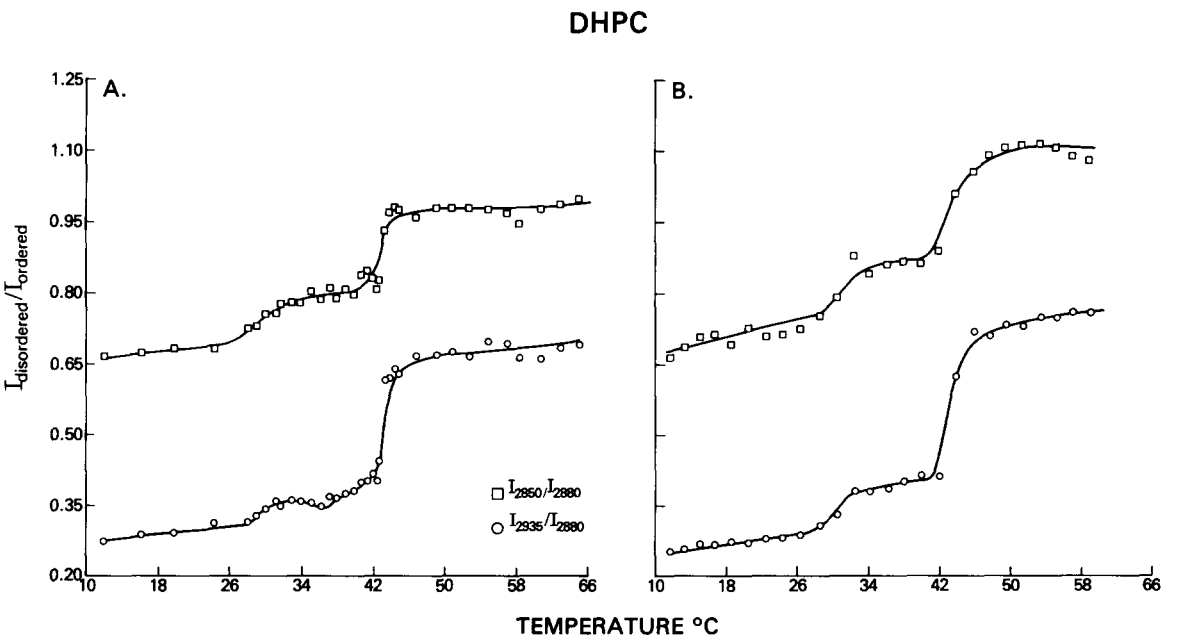

Fig. 2. Temperature profiles for DHPC dispersions in excess water. Raman spectral peak height intensity ratios $I(2935\ cm^{-1})/I(2880\ cm^{-1})$ (○) and $I(2850\ cm^{-1})/I(2880\ cm^{-1})$ (□) were used as indices to characterize the thermal behavior of the hydrocarbon chain region of the liposomes. The spectral data were obtained with a spectrograph using multichannel diode array detection. (A) temperature profiles for the more ordered form of the pure bilayer. (B) temperature profile for the disordered form of the pure bilayer.

sistent with that expected for interdigitated hydrocarbon chain systems [13,18], the 0.74 ratio for the disordered gel form (Fig. 1A) has been associated with a non-interdigitated bilayer [18]. It is interesting to note that gel-state forms whose low-temperature spectra are consistent with interdigitated bilayer species may, however, yield either the disordered liquid-crystalline polymorph (Fig. 2B, vide infra) or the more ordered, dehydrated liquid-crystalline form (Fig. 2A, vide infra). It thus appears that the tendency toward headgroup dehydration in the liquid-crystalline form is perhaps independent of the state of hydrocarbon chain interdigitation.

Since temperature profiles derived from Raman spectral intensity ratios obtained from the C-H stretching mode region conveniently describe the thermal behavior of the bilayer hydrocarbon chain region, we present several sets of temperature curves in Fig. 2 for dispersions manifesting varying amounts of bound water within the polar headgroup. These spectra were obtained with a spectrograph utilizing multichannel diode array detection techniques. Profiles constructed from both intensity ratios $I(2840\ cm^{-1})/I(2882\ cm^{-1})$ and $I(2934\ cm^{-1})/I(2882\ cm^{-1})$ are shown, with the profiles for the more disordered liquid crystalline assembly appearing in Fig. 2B. Regardless of the extent of headgroup hydration, the profiles exhibit the gel-to-liquid-crystalline phase transition T_m at 43°C. This value agrees with the differential scanning calorimetric value of 43.5°C [6]. The value obtained by Raman spectroscopy for the DHPC bilayer is slightly higher than the Raman value of 41.3°C obtained for DPPC bilayers, a system for which the hydrocarbon chains exhibit greater gel-state lattice disorder characteristics. For DHPC the pretransition appears approx. 1.4 Cdeg lower, however, for the more ordered gel state (29.4°C compared to 30.8°C, see Table I). The more ordered gel and liquid-crystalline phase forms of DHPC (Fig. 2A) manifest a significantly greater order than the analogous diacyl DPPC bilayers, as demonstrated by comparisons of the profiles at comparable reduced temperatures [10]. We emphasize that DPPC liposomes do not show changes, in particularly the liquid crystalline state disorder, as observed in DHPC dispersions, upon repetitive cycling through the phase transition. Hydrated DPPC bilayers, however, exhibit a lamellar crystalline, L_c, phase which is associated with a reduced headgroup hydration accompanying hydrocarbon chain ordering [19,20].

Since cholesterol containing DHPC bilayers yield the more disordered bilayer forms, we will compare the profiles for these systems to the pure DHPC profiles showing the maximum hydration effects. The temperature behavior for the cholesterol-containing dispersions are shown in Fig. 3 for profiles derived from both the $I(2934\ cm^{-1})/I(2882\ cm^{-1})$ and $I(2846\ cm^{-1})/I(2882\ cm^{-1})$ peak height intensity ratios. The presence of 18 mol% cholesterol in the DHPC bilayers disorders the gel state and orders the liquid-crystalline state in comparison to the pure DHPC dispersions (Fig. 3). As in the case of cholesterol-containing DPPC bilayers, the sterol also eliminates the DHPC bilayer pretransition. The latter effect perhaps arises as a consequence of the sterol spacer acting to alter or eliminate the chain tilt of the ether phospholipid in the gel state.

Although we prefer to associate the disordered gel and liquid-crystalline states of the cholesterol containing liposomes with an increase in headgroup bound water (vide supra), the increased lipid disorder could alternatively arise from the sterol's higher placement toward the headgroup of the bilayer. A perturbation of the headgroup region as, for example, a methyl substitution at the C(1) position of the glycerol backbone in the diether system, results in spectra reflecting a disordered lattice with increased numbers of *gauche* chain conformations (Lewis, N., Bittman, R. and Levin, I.W., unpublished data). Recent ^{13}C nuclear magnetic resonance and permeability studies, however, indicate that cholesterol occupies essentially the same region in membranes composed of either diacyl or diether lipids [5].

The C-H stretching mode region temperature profiles for the cholesterol containing DHPC bilayers, shown in Fig. 3, illustrate a dramatic difference between the effects of the sterol on diether lipid and on diacyl lipid dispersions. Cholesterol primarily broadens the transition for DPPC bilayers [1], although for 18 mol% cholesterol-containing dispersions, the Raman spectral data suggest a possible lowering of the pure DPPC phase transition by approx. 1 Cdeg (Levin and Bush,

TABLE I

SUMMARY OF THE THERMAL DATA FOR DHPC AND DHPC+18 MOL% CHOLESTEROL BILAYER SYSTEMS DERIVED FROM RAMAN SPECTRAL PARAMETERS.

Thermal data (T_m and ΔT) were determined from a least-squares fit to a two-state model for the gel to liquid crystalline phase transition. (Kirchhoff, W.H. and Levin, I.W., unpublished data.) T_m represents the main phase transition. ΔT represents the phase transition breadth. T_p represents the pretransition.

Bilayer	Raman marker	T_m (°C)	ΔT (Cdeg)	T_p (°C)
DHPC	$I(2935\ cm^{-1})/I(2880\ cm^{-1})$ [a]	42.8±0.2	2.5±0.5	30.8
	$I(2850\ cm^{-1})/I(2880\ cm^{-1})$ [a]	42.3±1.0	< 2.5	30.0
	$I(1090\ cm^{-1})/I(1130\ cm^{-1})$ [a]	44.1±0.2	< 2.8	30–33.5
DHPC+18 mol% cholesterol	$I(2935\ cm^{-1})/I(2880\ cm^{-1})$ [a]	49.6±0.4	14.0±1.4	
	$I(2850\ cm^{-1})/I(2880\ cm^{-1})$ [a]	49.8±1.0	14.0±1.5	
	$I(1090\ cm^{-1})/I(1130\ cm^{-1})$ [a]	48.8±1.8	16.9±2.9	
DHPC (ordered polymorph)	$I(2935\ cm^{-1})/I(2880\ cm^{-1})$ [b]	43.1±0.4	<1.4	29.4
	$I(2850\ cm^{-1})/I(2880\ cm^{-1})$ [b]	43.2±0.4	<1.4	29.4
DHPC (disordered polymorph)	$I(2935\ cm^{-1})/I(2880\ cm^{-1})$ [b]	43.0±1.0	< 3.4	30.8
	$I(2850\ cm^{-1})/I(2880\ cm^{-1})$ [b]	43.0±1.0	< 3.8	30.8

[a] Raman spectral data obtained on a scanning monochromator with photomultiplier detection.
[b] Raman spectral data obtained on a spectrograph with diode array detection.

unpublished data, see Ref. 7). In contrast, the 18 mol% sterol concentration in the lipid DHPC bilayers increases the phase transition temperature by approx. 7 Cdeg from 42.8°C to 49.6°C. These data are also summarized in Table I. This increase in T_m suggests either a discrete sterol-ether lipid complex or a long-lived associated species. The broadening of the phase transition region for the sterol containing systems indicates a decrease in the cooperativity of the lattice and chain disordering phenomena, which may imply a slow dissociation of the sterol-lipid complex. The ability of the sterol to form a more tightly associated complex in the ether lipid bilayer, in comparison to acyl systems, arises from the increase in van der Waals interactions between chains when the carbonyl groups are eliminated. Presti et al. have postulated for diacyl lipids a colinear hydrogen bond between the 3β-OH group of cholesterol and the esterified glycerol oxygen of the *sn*-2 chain [1]. For the diether lipids, elimination of the carbonyl groups increases the flexibility of bond rotations within the interface region [21], which may indeed favor the possibility of a strong hydrogen bond between the ether linkage and the OH group of the sterol. It would be of interest to examine sterol-containing bilayer systems for which the chains reflect mixed acyl and ether links in order to elucidate further the effects of the putative ether hydrogen bond.

Although the cholesterol complexes formed with the saturated, diacyl phosphatidylcholine bilayers generally only broaden the gel to liquid crystalline phase transition, complexes of cholesterol and lanosterol with unsaturated lipid bilayers composed of 1-palmitoyl-2-oleoyl phosphatidylcholine increases T_m by approx. 8 and 4.5 Cdeg, respectively (Ref. 7 and Levin and Bush, unpublished data). The increase in T_m by cholesterol in this case was attributed to maximizing the van der Waals contacts between the sterol and the acyl chain pocket formed by the C9-C10 *cis* double bond in the 2-oleoyl chain of 1-palmitoyl-2-oleoylphosphatidylcholine [7].

The carbon-carbon stretching mode region of the hydrocarbon chains reflects intrachain *trans/gauche* isomerization due either to temperature changes or to bilayer-disrupting perturbations [7,8]. Fig. 4 and Table I summarize the intrachain order/disorder data for the pure DHPC- and cholesterol-containing bilayers. Temperature profiles constructed from either $I(1090\ cm^{-1})/I(1130$

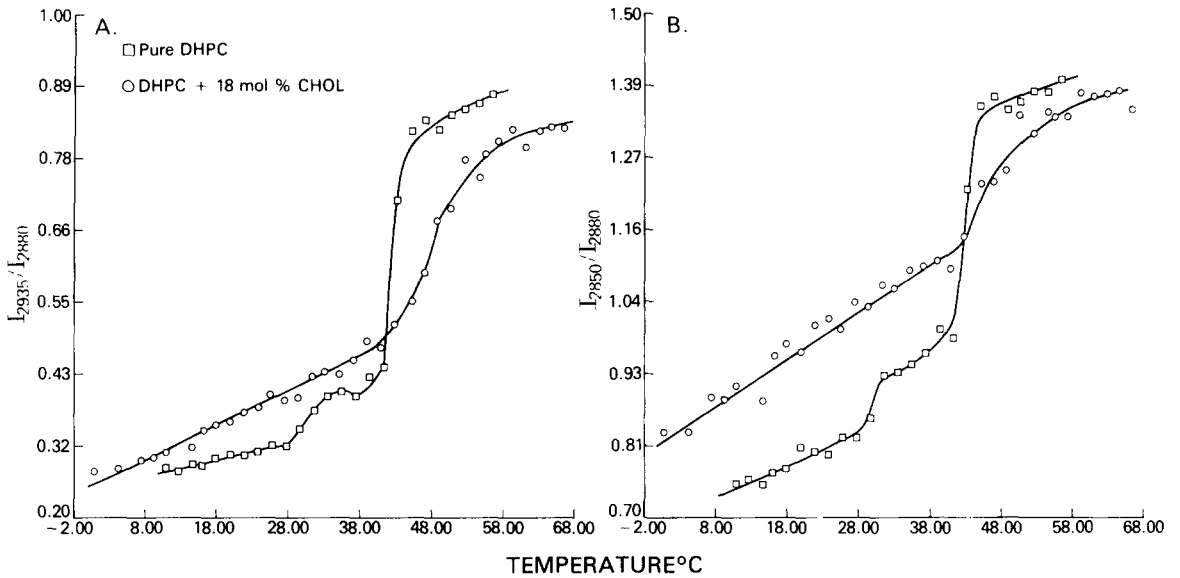

Fig. 3. Temperature profiles for pure DHPC (□) and DHPC + 18 mol% cholesterol (○) liposomes using the Raman spectral $I(2935\ cm^{-1})/I(2880\ cm^{-1})$ (A) and $I(2850\ cm^{-1})/I(2880\ cm^{-1})$ (B) peak height intensity ratios as indices.

cm^{-1}) or $I(1090\ cm^{-1})/I(1060\ cm^{-1})$ peak intensity ratios are consistent with the spectral data obtained from the C-H stretching mode region. The scatter of points in the liquid-crystalline phase is somewhat greater in comparison to the C-H stretching mode region profiles. This results from the intransically weaker C-C stretching mode signals. Although the assignments for the C-C stretching modes have also been discussed in detail previously, we note that the approx. 1090 cm^{-1} feature arises directly from the introduction of *gauche* conformers into an all-*trans* chain segment

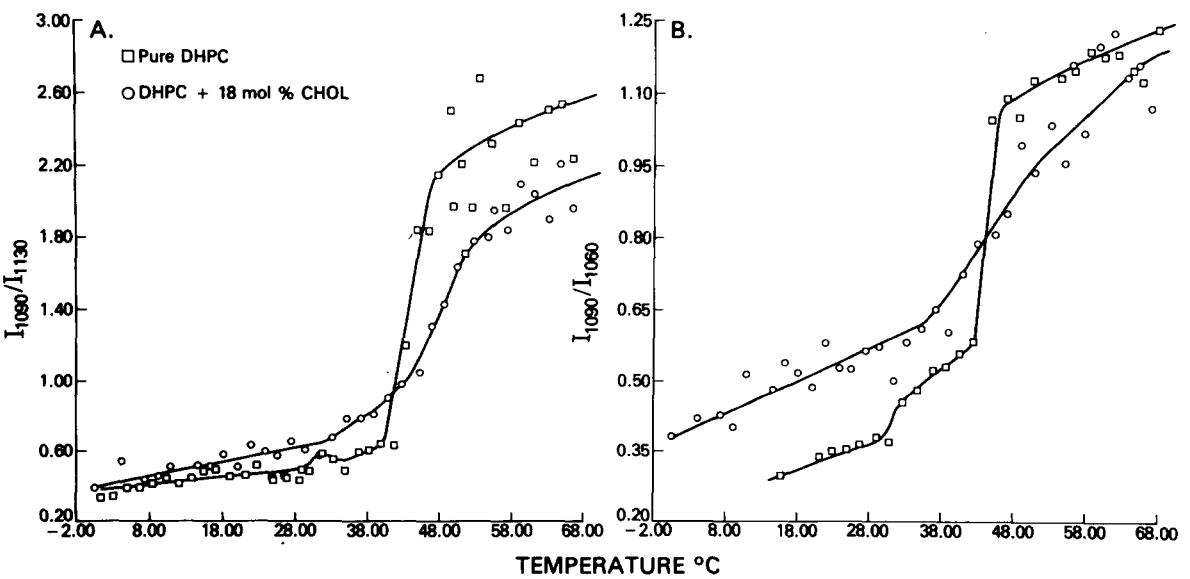

Fig. 4. Temperature profiles for pure DHPC (□) and DHPC + 18 mol% cholesterol (○) liposomes using the *I(gauche)/I(trans)* peak height intensity ratios as indices. (A) temperature profile using $I(1090\ cm^{-1})/I(1130\ cm^{-1})$ intensity ratios. (B) temperature profiles using the $I(1090\ cm^{-1})/I(1060\ cm^{-1})$ intensity ratios.

[7]. As given in Table I, ΔT, a measure of the cooperativity of the chain melting process, appears slightly greater for the intrachain disordering process ($\Delta T = 16.9 \pm 2.9$ Cdeg), in comparison to the temperature interval reflecting primarily a lattice subcell reorganization ($\Delta T = 14.0 \pm 1.5$ Cdeg). Since all parameters reflecting the bilayer temperature behavior were obtained from a least-squares fit to a two-state model (Kirchhoff, W.H. and Levin, I.W., unpublished data), the uncertainties in ΔT suggest that the perceived differences in cooperativity between the lattice and intrachain disordering characteristics may not be meaningful.

In summary, the thermal behavior of aqueous dispersions of saturated chain diether lipid bilayers containing 18 mol% cholesterol differs significantly from the observations noted for cholesterol dispersed in saturated chain diacyl systems. Although the sterol disorders the gel state while ordering the liquid-crystalline phase, in both diether and diacyl aqueous dispersions, cholesterol increases the phase transition temperature T_m by nearly 7 Cdeg in the diether assembly; in saturated chain diacyl systems cholesterol only broadens the transition about T_m. In addition to increasing T_m in DHPC bilayers, cholesterol reduces the cooperativity of the phase transition process. In contrast to aqueous dispersions of diacyl bilayer systems, DHPC, dispersed in water, exhibits an interdigitated gel state [17] and a gel and liquid crystalline polymorphic behavior based upon a headgroup dehydration effect. That is, as pure DHPC bilayers are repetitively cycled through their gel and liquid crystalline phases, the liquid crystalline liposomes exhibit an increasingly ordered hydrocarbon chain region. In this context, melted anhydrous polycrystalline DHPC bilayers exhibit only the 'more ordered' lattice forms.

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