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LATERAL INTERACTION OF CHOLESTEROL IN DIACYLPHOSPHATIDYLCHOLESTEROL BILAYERS

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Thermotropic phase-transition properties of the aqueous dispersions of several diacylphosphatidylcholesterol (DRCh) analogs are examined. The aqueous dispersions of their calcium salts exhibit characteristic endothermic thermotropic transitions due to a change in the conformation of acyl chains. These dispersions consist of osmotically intact liposomes that trap ions, and at the transition temperature there is anomalous increase in the ion leakage. Wide-angle electron diffraction studies of DPCh · Ca monolayers also exhibit a transition from a sharp 4.25 Å band to a broad one centering at 4.7 Å, reflecting an order-disorder transition in the acyl chains. The long-range order in the organization of acyl chains of DRCh molecules could arise from intermolecular interactions between the cholesterol moieties to form a functional dimer, and such dimers are apparently cross-linked by Ca^{2+} to form a long-range interacting lattice of acyl chains. Evidence for this model is adduced from the fluorescence properties of the dispersions of dimyristoylphosphatidylcholesta-5,7,9-trienol. The phase-transition properties of DRCh are an ideal illustration of calcium-induced isothermal phase transition.

Introduction

Dipalmitoylphosphatidylcholesterol (DPCh), a covalent analog of an equimolar mixture of dipalmitoylphosphatidic acid and cholesterol, forms bilayer-enclosed liposomes in aqueous dispersions [1,2]. In the presence of calcium ions, the DPCh bilayers exhibit a sharp endothermic transition which is accompanied by a change in the anisotropy of polarization of diphenylhexatriene [2]. The phase-transition temperature and the enthalpy of this transition depend upon acyl chain-length, that is, they decrease with decreasing chain-length. In contrast, the bilayer formed by equimolar noncovalent mixtures of diacylphospholipids and cholesterol do not exhibit phase transitions analogous to those observed in DPCh bilayers [3–5].

The cooperativity of the thermotropic transition in the much studied diacylphosphatidylcholine dispersions implies that there exists a long-range interaction between the acyl chains in these bilayers. In the present work, we have examined the consequences of such an ordered state of the acyl chains in bilayers of several analogs and homologs of DRCh. The wide-angle electron diffraction studies, the thermotropic phase transition and the ion-permeability characteristics of their aqueous dispersions exhibit a thermotropic dependence that is consistent with a high cooperativity of the interact-

Abbreviations: DRCh, diacylphosphatidylcholesterol; DPPC/DMPC, dipalmitoyl/dimyristoylphosphatidylcholine; DRChene, diacylphosphatidylcholesta-5,7,9-trienol; R = M, myristoyl; P, palmitoyl; S, stearoyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ing state of the acyl chains. This implies that there should be considerable intermolecular interaction between cholesterol moieties, since such an interaction is required for the long-range interaction between acyl chains in DPCh and its analogs. Evidence for an interaction between the cholestane moieties is described in this paper.

Materials and Methods

DRCh was synthesized as previously described [6]. Diacylphosphatidylhexadecanol, diacylphosphatidyloctadecanol and diacylphosphatidylcyclododecanol were prepared from the respective alcohols by a similar procedure [6]. Diacylphosphatidylcholesta-5,7-diene-3β-ol and diacylphosphatidylcholesta-5,7,9-triene-3β-ol (DRChene) were synthesized as described [7]. The preparation of cholesta-5,7,9-triene-3β-ol phosphate has also been described [8]. According to the usual criteria, the fluorescent derivatives used in this study have no detectable impurities [6–8]. Other phospholipids were purchased from Calbiochem, and judged to be homogeneous by thin-layer chromatography.

The fluorescence measurements were done on an SLM4800 spectrofluorometer equipped with lifetime and phase-resolved spectrum modules. Excitation and emission slit-widths were 4 and 4, and 4 and 8 nm for the steady-state and for the dynamic measurements, respectively. Standard software package for SLM4800S was used for data acquisition and processing. All the spectra reported here are uncorrected. The solutions for fluorescence measurements contained 190 μM DRChene with appropriate concentrations of additives in 100 mM KCl/10 mM Hepes/10 mM $CaCl_2$ (pH 8.0). Steady-state measurements were done in ratiometric mode. Lifetime measurements were done by using POPOP ($\tau = 1.35$ ns) as a reference standard [9]. Phase-sensitive spectra were obtained by modulating the detector phase angle at 6 and 30 MHz [10].

Dispersions of phospholipids were prepared by suspending a dry film of the phospholipids in aqueous buffer at about 70 °C. Occasionally, sonication in a bath-type sonicator was required for complete dispersal of the lipids. Lipids containing fluorescent analogs were dispersed in the buffer saturated with nitrogen. For permeability studies, the dispersions were prepared and ^{22}Na effluxes were measured as described elsewhere [11]. Differential thermal analysis or DSC studies were done on a Mettlar 2000B or Perkin-Elmer 1B calorimeter [1,2].

Electron-diffraction studies were done on monolayers. This method has two major advantages: it can examine a single unsupported bilayer prepared by the monolayer method [12] and it can examine a small area (0.5 μm in diameter) to detect any local structural variation. The procedures of electron diffraction are described by Hui and He [13]. It should be pointed out that the electron diffraction experiments were carried out in an environmental stage within the electron microscope [14] with the sample fully hydrated at controlled temperatures.

Results

The thermotropic phase-transition characteristics of several analogs of DRCh are summarized in Table I. The T_m increases with increasing acyl chain-length. T_m is also affected by the number of double-bonds in the cholesterol moiety. The replacement of cholesterol by cyclododecanol in these lipids lowers T_m and broadens the transition profile considerably. On the other hand, diacylphosphatidylhexadecanol and the homologous octadecanol give sharp transition. Thus, the transition behavior is also affected by the structure of the second group of the phosphodiester. These observations demonstrate that the transition behavior of these compounds is due to cooperative intermolecular interactions and conformational changes in the acyl chain regions.

As demonstrated by freeze-fracture electron microscopy and osmotic swelling, DPCh dispersions form osmotically sensitive liposomes [1] that are impermeable to ions. Fig. 1 shows that the trapped ions leak out more rapidly at the phase-transition temperature of these dispersions than above or below that temperature. This anomalous increase in ion leakage provides an independent confirmation of changes in the acyl chain organization during the thermotropic transition. The ion-permeability profiles presented in Fig. 1 demonstrate that the temperature-dependent anomalous in-

TABLE I

PHASE TRANSITION CHARACTERISTICS OF THE AQUEOUS DISPERSIONS OF DRCh AND ITS ANALOGS

The values in the parentheses are for the aqueous dispersions of the sodium salt. In all other cases a transition could not be detected for the dispersions of the sodium salt.

Compound (calcium salt)	T_m (°C)	ΔH (kcal/mol)
Dilauroylphosphatidylcholesterol	35	5
Dimyristoylphosphatidylcholesterol	38	7
Dipalmitoylphosphatidylcholesterol	47	8
Distearoylphosphatidylcholesterol	50	9.5
Dipalmitoylphosphatidylcholesta-5,7-diene	55	8.5
Dipalmitoylphosphatidylcholesta-5,7,9-triene	43	8
Dimyristoylphosphatidylcholesta-5,7,9-triene	35	6.8
Dipalmitoylphosphatidylhexadecanol	47 (70)	12.4 (8.7)
Dipalmitoylphosphatidyloctadecanol	51 (75)	15.3 (10.0)
Distearoylphosphatidylcyclododecanol	22	6.

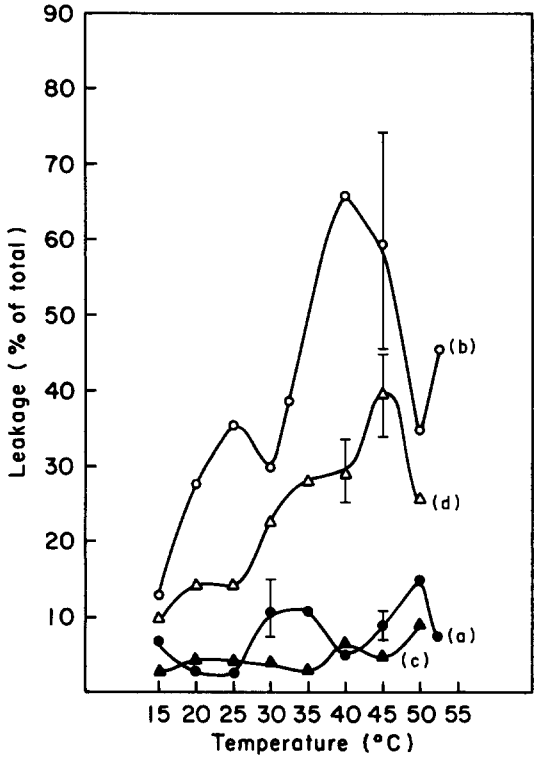

Fig. 1. Permeability profiles for DPCh as a function of temperature. The phospholipid was only available in the form of its calcium salt for these experiments. (a) Vesicles dispersed in 5 mM NaCl/5 mM Tris-HCl/20 mM EDTA/150 mM glucose/^{22}NaCl (pH 7.5) by sonication with a probe-type sonicator at 0 °C. (b) Vesicles dispersed in 5 mM NaCl/5 mM Tris-HCl/20 mM EDTA/50 mM $CaCl_2$/^{22}NaCl (pH 7.5) by sonication with a probe-type sonicator at 0 °C. (c) Unsonicated vesicles dispersed in 5 mM NaCl/5 mM Tris-HCl/20 mM EDTA/150 mM glucose/^{22}NaCl (pH 7.5). (d) Unsonicated vesicles prepared as in (c) but with calcium chloride then added to the outside of the performed vesicles. Each experimental point represents a separate experiment performed in triplicate. Datum points are means and error bars that have been added to some of the points to illustrate the range of values obtained. The ordinate refers to the percent loss of initial trapped ^{22}Na in 180 min.

crease in permeability is seen only in the presence of calcium ions on one or both sides of the bilayer. We considered the possibility that the anomalous peak in ^{22}Na efflux rate for DPCh dispersions in the presence of calcium ions could be secondary to aggregation and subsequent fusion of vesicles and not due to a true change in permeability. Vesicle aggregation was measured by absorbance at 450 nm. Absorbance remained constant throughout the time period of efflux measurements. This observation was true for all the temperatures at which ^{22}Na effluxes were examined.

We also measured the permeability versus temperature profiles for dispersions formed from dipalmitoylphosphatidylhexadecanol or distearoylphosphatidylcyclododecanol (data not shown). The former displayed a calcium-dependent permeability maximum at about 50 °C, whereas the barrier properties of distearoylphosphatidylcyclododecanol were independent of calcium.

The temperature dependence of wide-angle electron diffraction patterns of DPCh was measured. Even as the electron beam diameter was reduced to 1 μm to examine any locally oriented domains of acyl chains, no azimuthal asymmetry

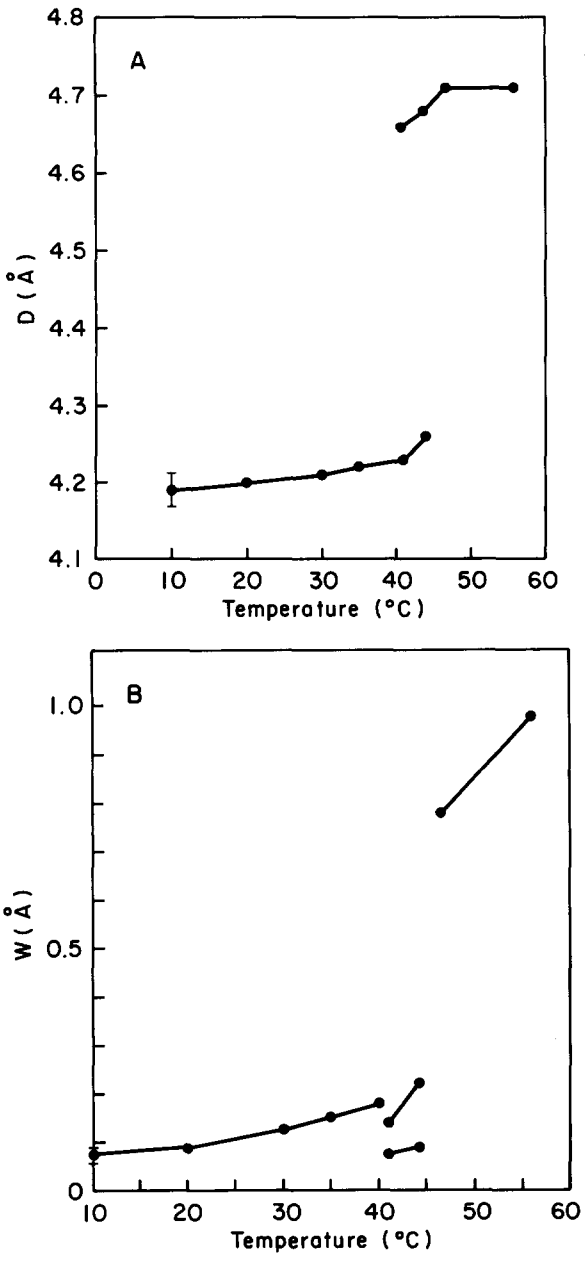

Fig. 2. The temperature dependence of the electron diffraction intensities of DPCh·Ca monolayer: (A) peak position, D, and (B) peak half-width, W.

of diffraction pattern was observed. This is in contrast to the mixtures of DMPC and cholesterol which show a two-field symmetric pattern suggestive of a unidirectional alignment of acyl chains in local area [13]. Fig. 2 shows the temperature dependence of the wide-angle electron diffraction of the calcium salt of DPCh. The result is markedly different from that of the calcium-free DPCh (data not shown). At $T < 40\,^{\circ}C$, the calcium salt shows a gradual increase in diffraction spacing and peak width, W, with increasing temperature. At $40\,^{\circ}C < T < 47\,^{\circ}C$, two wide-angle reflections co-exist, a sharp one at 4.25 Å and a broad one centering at 47 Å. This is typically a phenomena of solid/fluid phase separation. The phase separation lasts until the temperature reaches $47\,^{\circ}C$ when only one broad reflection at 4.7 Å remains, signalling that the phase transition is completed. Again no azimuthal asymmetry is detected at any temperature range.

The fact that DPCh · Ca shows phase transition, whereas calcium-free DPCh or mixture of DPPC and cholesterol do not, indicates the linkage between the calcium, diacylglycerolphosphate and covalently bonded cholesterol favors a long-range cooperative organization of these molecules. Moreover, the fact that a definite phase-separation range exist in DPCh · Ca suggests that larger domains of strong molecular order, similar to that in a gel-phase lipid, exist in these dispersions. This effect of calcium on DPCh is analogous to the effect of the same cation on phosphatidylserine [15]. It is interesting to note that as the temperature rises, the sharp reflection at 4.2 Å of DPCh · Ca is gradually broadened to the onset of the phase-separation region, then it is sharpened abruptly. If the line-broadening is due to the thermal disorder, this observation indicates that as the temperature rises, the packing of the acyl chains in the calcium salt is generally loosened and the molecules are more free to move until phase separation occurs.

From the thermotropic phase change in bilayers of the calcium salt of DRCh, we infer that there could be significant intermolecular interactions between the cholesterol moieties in these compounds in such a way as to permit a long-range lateral interaction between acyl chain with the consequent high cooperativity of the transition. To scrutinize this effect we have studied the fluorescence properties of aqueous dispersions of DRChene. Rogers et al. [16] have already studied the properties of diacylphosphatidylcholine bilayers containing cholesta-5,7,9-triene-3β-ol and

ergosta-5,7,9-triene-3β-ol. Their results show that the properties of bilayers containing these fluorescent analogs are very similar to those of diacylphosphatidylcholine bilayers containing cholesterol.

The absorption spectra of dimyristoyl- (DMChene) and dipalmitoylphosphatidylcholestatrienol (DPChene) in hexane are very similar to the spectra of cholestatrienol and its dihydrogen phosphate in the same solvent; these spectra had peaks at 286, 312, 331 and 346 nm. Similar values were obtained for DMChene in hexane tetrahydrofuran and anhydrous chloroform (data not shown). Excitation at any of these wavelengths gives the same fluorescence emission spectra the intensities of which are approximately in the same proportion as the absorption intensities. Fig. 3, shows the emission fluorescence spectra of DMChene in hexane and in aqueous dispersions with peaks at about 370 and 390 nm. The exact position of these peaks and their absolute and relative intensities depend upon the exact conditions, including temperature, presence of divalent ions and other additives in the dispersions as can be seen from Table II. The ratios of the two emission peaks are independent of the wavelengths of the excitation peaks. For all the studies reported in this paper, an excitation wavelength of 326 nm was used. Although not shown, essentially identical results were obtained with DPChene. In all cases, we observed that the absolute and relative intensities of the two emission peaks are very sensitive to both the aqueous environment and the environment in the bilayer itself. It may be pointed out that the emission spectra of cholestatrienol and cholestatrienol phosphate exhibit only a single broad peak. Thus, the two peaks in DRChene spectra in solution as well as in dispersions could be due to the anisotropic environment induced by the acyl chains.

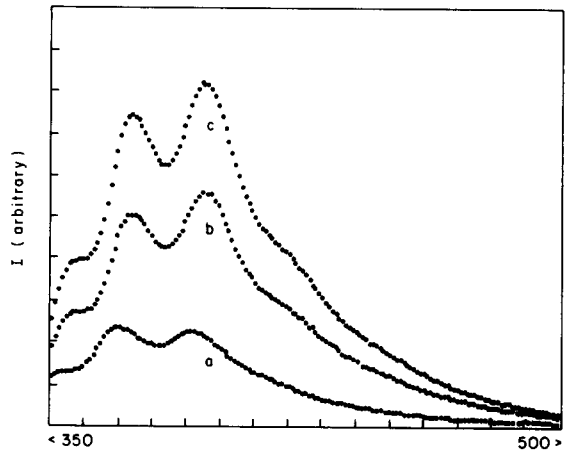

Fig. 3. Excitation and emission spectra of 190 μM DMChene in (a) hexane, (b) aqueous dispersions containing calcium and (c) aqueous dispersions containing no calcium. Excitation at 326 nm, slit-widths at 4 nm for both excitation and emission monochromators.

One of the major effects of the presence of calcium in the aqueous environment is a decrease in the intensities of both the emission peaks, while the ratio of their intensities remains the same (Table II). The ratio of the intensities in the difference spectrum is also the same. The Scatchard plot of the decrease in intensity with the concentration of calcium in the medium, is shown in Fig. 4. It exhibits two binding processes of K_d 0.1

TABLE II

EMISSION SPECTRAL PROPERTIES OF DMChene UNDER VARIOUS CONDITIONS

Unless stated otherwise, all solutions contained 190 μM DMChene. The aqueous buffer comprised 100 mM KCl/10 mM Hepes/10 mM $CaCl_2$ (pH 8.0). Excitation at 326 nm, slit-widths at 4 nm for both excitation and emission monochromations.

Conditions	Peak 1, nm (intensity)	Peak 2, nm (intensity)	Ratio (I_1/I_2)
Hexane, 25 °C	369 (0.2324)	390 (0.2211)	1.051
Buffer, 25 °C	374 (0.5017)	394 (0.5526)	0.9079
Buffer, 38 °C	374 (0.2985)	394 (0.3044)	0.9806
Ca-free buffer, 25 °C	374 (0.7379)	393 (0.8143)	0.9062
Ca-free buffer, 38 °C	374 (0.5337)	394 (0.5511)	0.9684
Codispersed with DMPC, 25 °C	374 (0.8928)	393 (0.8906)	1.0024
Codispersed with DMCh, 25 °C	374 (0.7289)	394 (0.7047)	1.034

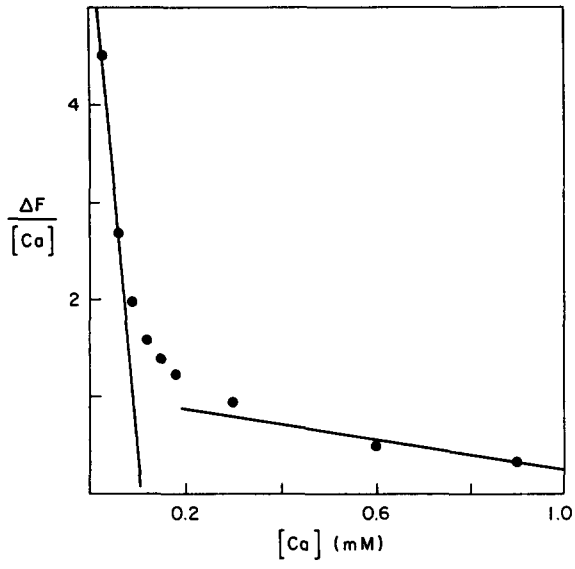

Fig. 4. Scatchard plot of decrease in fluorescence intensity ($^{-}\Delta I$) of DMChene at 394 nm as a function of calcium concentration. Other conditions are givenin the legend of Fig. 3.

and 1.3 mM. The significance of which is not clear to us at this stage.

The calcium-induced decrease in fluorescence intensity is presumably due to self-quenching brought about by intermolecular bridging by calcium bound to the phosphate groups. This is best illustrated by an increase in the fluorescence intensity of DMChene dispersions on surface dilution with other lipids. As shown in Fig. 5, codispersions of DMChene with varying mole fractions of DMCh or DMPC lead to an increase in the fluorescence intensity. By extrapolation of the linear region, it can be calculated that the fluorescence intensity of completely unquenched DMChene at infinite dilution would be about 3.5-times more than that of the DMChene vesicles. While the fluorescence intensity increases linearly at low mole fractions of the nonfluorescent additives, it decreases at higher mole fraction of the additives. We believe that this decrease in intensity is due to turbidity from the higher total lipid concentration used for surface dilution. In these

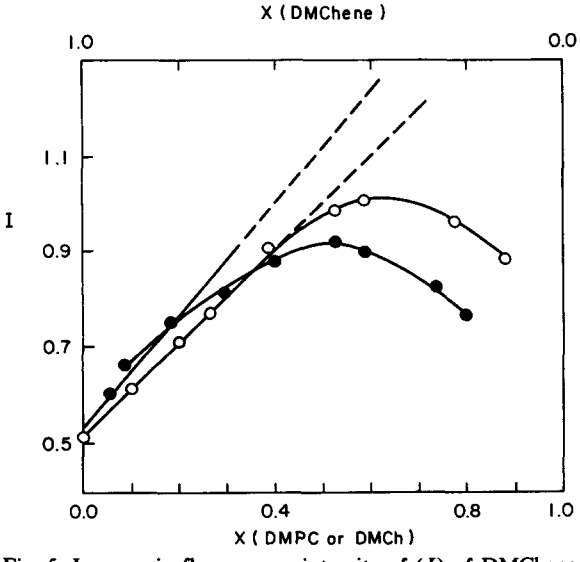

Fig. 5. Increase in fluorescence intensity of (I) of DMChene (190 μM) at 3904 nm in dispersions containing varying mole fractions (×) of DMPC (○) and DMCh (●). All solutions contained 10 mM $CaCl_2$. Other conditions are given in the legend to Fig. 3. Plots of the emission intensities at 373 nm gave virtually identical plots.

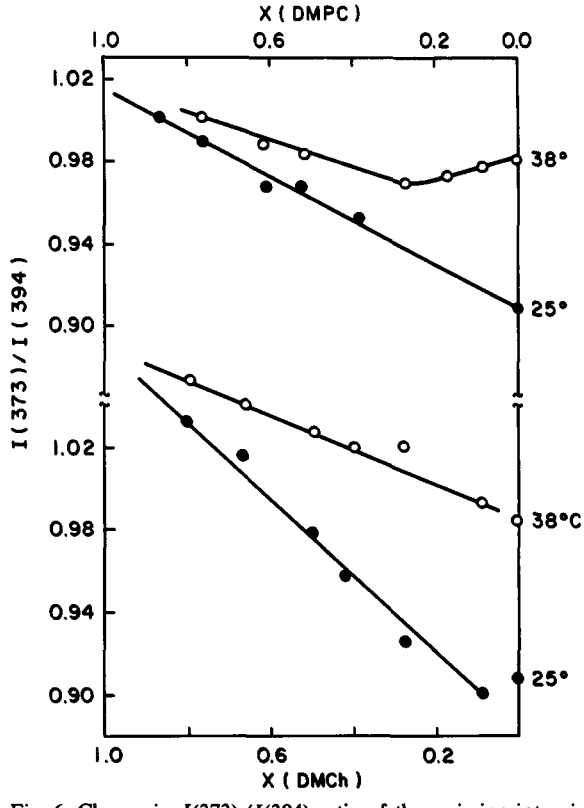

Fig. 6. Change in $I(373)/I(394)$ ratio of the emission intensities of DMChene (190 μM) containing varying mole fractions of DMPC (top) and DMCh (bottom) at the temperatures indicated.

measurements, the probe concentration is kept constant while varying its mole fraction.

One of the important differences in the emission spectrum of the surface-diluted DMChene vesicles and that of the undiluted vesicles is that the relative intensities of the peaks at 372 and 394 nm are different. By using the polarizing and the cut-off filters, by changing the slit-width, and by using a mixture of the dispersions to increase the turbidity without surface dilution, it was established that the change in the ratio of the intensities of the two peaks is not an artifact of scattering of the excitation beam by the turbid solutions. In fact, as summarized in Table II, the $I(373)/I(394)$ ratio is 1.051 in hexane and 0.9079 in aqueous dispersions of 190 μM DMChene and both of these solutions had no noticeable turbidity. As shown in Fig. 3, the absolute intensities are also quite different. Based on such controls, and other experiments described below, we believe that the ratio of the intensities at 373 and 394 nm is a useful measure of the intermolecular interaction between DMChene molecules in a bilayer, and this ratio does not suffer from many of the artifacts that influence the fluorescence intensity values. As shown in Fig. 6, the $I(373)/I(394)$ ratio increases with increasing mole fraction of DMCh or DMPC in the mixed codispersions with DMChene at 25 and at 38°C. While the intensity ratio increases linearly with the mole fraction of the additive lipid, a somewhat anomalous behavior is observed at 38°C with the codispersions containing DMPC + DMChene. Little or no change in the intensity ratio is seen at low mole fractions of DMPC, suggesting that in these bilayers, the affinity of DMChene for itself is greater than it is for DMPC. This could lead to lateral phase separation. The linearity of the other three curves in Fig. 6 suggests an ideal mixing of the two lipid components under these conditions.

Insight into the mechanism of self-quenching of DMChene in a bilayer can be obtained by the measurement of fluorescence lifetimes. Both the pulse method and the phase method suggest fluorescence lifetime heterogeneity [17,19,20]; therefore, these results should be taken only as a qualitative guide. As summarized in Table III, the lifetime of DMChene in hexane obtained by the phase-shift method, τ_p, is about 0.43 ns, which

TABLE III

FLUORESCENCE LIFETIME OF DMChene AT 25°C

Conditions	τ_p (ns)		τ_m (ns)	
	30 MHz	6 MHz	30 MHz	6 MHz
In hexane	0.43	0.72	2.45	7.9
In buffer without Ca	1.58	1.89	2.53	4.9
In buffer + Ca	1.77	2.3	3.64	7.7
Codispersed Ca + DMCh (1 : 3)	1.88	2.6	3.41	7.4
Codispersed Ca + DMPC (1 : 3)	1.77	1.80	2.46	3.5

compares favorably with the value of 0.53 ns obtained by the pulse method. A difference in the τ_m and τ_p values at 30 and 6 MHz, as well as the decay curve in the pulse method show the presence of two or more components. In the aqueous dispersions of DMChene, the lifetimes increase to about $\tau_p \approx 1.5$–1.8 ns. It should, however, be emphasized that the lifetimes of DMChene in the various aqueous dispersions are much closer to each other than their fluorescence intensities. Such a lack of correlation between the fluorescence lifetimes and the fluorescence intensities, suggests that the self-quenching of DMChene is due to formation of a complex between the quencher and the fluorophore in the excited state [17]. Since both the quencher and the fluorophore are the same moieties in this case, it would imply that the two cholesterol moieties should be able to come in contact with each other during the lifetime of the excited state. Use of other membrane-localized quenchers could lead to further characterization of this complex.

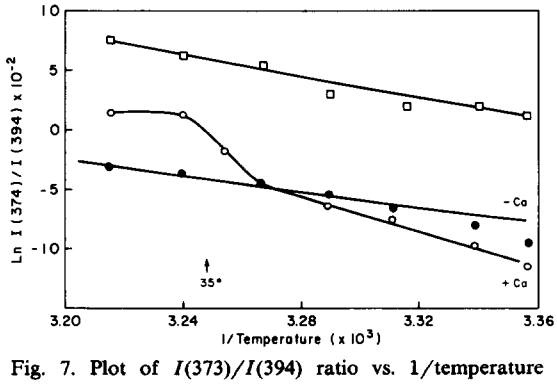

Fig. 7. Plot of $I(373)/I(394)$ ratio vs. 1/temperature for aqueous dispersions of DMChene (190 μM) without calcium (●) and with calcium (○), and codispersed with DMPC (□). Other conditions are given in the legend to Fig. 3.

An intermolecular interaction between the cholestatriene moieties in DMChene dispersions is also modified by temperature. As shown in Fig. 7, only the dispersions of DMChene · Ca exhibit a sharp increase in the $I(373)/I(394)$ ratio at 33°C, that is, at the same temperature at which the transition is seen by calorimetry (see Table I). Interestingly, such a transition is not seen with DMChene in the absence of calcium or in the codispersions of DMChene + DMPC in the presence of calcium.

Discussion

In this paper, we have supplemented our earlier observation [1,2] that the aqueous dispersions of the calcium salt of DRCh exhibit an endothermic phase transition. This system is of considerable theoretical interest because a transition of acyl chains is seen in a bilayer in which cholesterol and acyl chains are packed in 1 : 2 mole ratio. Also the fact that the transition is seen only in the presence of calcium ions, makes it an ideal example of solute-induced isothermal phase transition. In all other known examples of isothermal phase transition, a solute simply shifts the phase-transition profile.

The enthalpy and the temperature of this transition depend upon the acyl chain-length, suggesting that the endotherm is due to a cooperative order-disorder transition of the acyl chains. This hypothesis has now been directly corroborated with the electron diffraction measurements on the monolayer of DMCh · Ca. The high cooperativity of this transition raises several questions concerning the lateral organization of the acyl chains in the plane of the bilayer. One of the possibilities we suggested earlier is that an extensive interaction between the acyl chains, leading to a highly cooperative transition, is attained by one-dimensional/linear organization of the acyl chains. Such an arrangement implies an interaction between the cholesterol moieties. The fluorescence properties of DMChene unequivocally demonstrate that an interaction between the cholestatriene moieties does indeed lead to self-quenching. The change in the $I(373)/I(394)$ ratio as a function of surface dilution and temperature suggests that the orientation and motional freedom of the dipoles of the fluorophore are somehow involved in this phenomenon.

The self-quenching of DMChene is not accompanied by a significant decrease in the fluorescence lifetime; therefore, the static self-quenching of DMChene in the dispersions must be due to intermolecular interaction during the excited state. This implies that the fluorophores are probably very close to each other so that they can interact during the lifetime of the excited state. A precise arrangement of the DMCh molecules in the bilayer cannot be described yet. However, it should be noted that while formation of the calcium complex leads to the self-quenching without a change in the $I(372)/I(394)$ ratio, the effect of the surface dilution is exerted both on the absolute and relative intensities of the two peaks. It appears that the relative intensities of the two peaks arise from the dipole along one face of the cholestatriene moiety, whereas the change in the absolute intensity could be due to the interactions along one of the other faces of cholesterol and/or rotational freedom of the molecules.

It may be appropriate at this stage to relate the significance of our observations to the state of cholesterol in phospholipid bilayers. Several laboratories have reported specific cholesterol-cholesterol and cholesterol-lipid interaction in bilayers. This is necessary, not only because of the geometrical constraints of packing beyond 30 mol% cholesterol in codispersions with diacylphospholipids, but also because there is substantial evidence that cholesterol tends to cluster in stoichiometric ratios with diacylphospholipids even when it is present at low mole fractions in codispersions [16,21–26], cf. discussion in Ref. 22). Several models have been invoked to account for such intermolecular interactions and the lateral organization of the components of these mixed bilayers [16,21–26]. For example, linear packing arrangement of cholesterol molecules has been proposed to interpret data from a variety of techniques. These models imply significant long-range chain-chain interaction. We have obtained direct evidence for such chain interactions by demonstrating thermotropic phase transition in aqueous dispersions of calcium salts of DRCh and its analogs, and from their fluorescence properties.

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