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Effects of cholesterol on membrane surfaces as studied by high-pressure fluorescence spectroscopy

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Abstract

We have studied the effects of cholesterol on membrane surfaces using fluorescence spectroscopy at high pressure. At atmospheric pressure, the dissociation state of a pH-sensitive fluorophore (6-decanylnaphthol or DECNA) incorporated into several types of membranes showed an apparent increase in dissociation with cholesterol content coming somewhat closer to its dissociation state in solution. Previous studies have shown that when DECNA is free in solution, pressure induces proton dissociation due to the volume decrease that occurs when water electrostricts around the ions. But in phosphatidylcholine (PC) bilayers, proton dissociation is inhibited, either due to the inability of the surface to expand and allow for increased hydration, or other changes in lipid structure. The pressure behavior of DECNA in dioleoyl-PC, dioleoylphosphatidic acid and dioleylphosphatidylglycerol bilayers shows that incorporation of 5–10% cholesterol causes DECNA to behave like it is in a more unrestricted environment. This trend is reversed at higher cholesterol concentrations. These data, together with compressibility measurements, support the model of Sankaram and Thompson [M. Sankaram, T.E. Thompson, Biochemistry 29 (1990) 10676.] whereby in the disordered phase, cholesterol can span the two leaflets causing an increase in the area between the head groups; whereas in the ordered phase, no expansion occurs. Thus, the effect of cholesterol on membrane surfaces depends on its phase diagram. © 1997 Elsevier Science B.V.

Keywords: Cholesterol; Membrane surfaces; Fluorescence spectroscopy; High pressure; Compressibility

1. Introduction

Cholesterol is the major sterol component in most mammalian plasma membranes and can be found in concentrations in excess of 30% (see Refs. [1,2] for review). Cholesterol is composed of a planar fused ring system with a 3β hydroxyl group and an alkyl side chain at position C17. There have been many studies on the molecular properties of cholesterol and its effects on membrane properties. Several func-

Abbreviations: AS: Anthroyloxystearic acid; DECNA: 6-Decanoylnaphthol; DMPC: Dimyristoylphosphatidylcholine; DOPA: Dioleoylphosphatidic acid; DOPC: Dioleolylphsophatidylcholine; DOPG: Dioleoylphosphatidylglycerol; Laurodan: 6-Lauronyl-(*N,N*-dimethylamino)naphthalene; PE: Phosphatidylethanolamine; OG: β-Octylglucoside; SDS: Sodium dodecylsulfate; suvs: Small, unilamellar vesicles; mlvs: Multilamellar vesicles.

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tional roles for cholesterol have been postulated, including reducing water permeability through the membrane (e.g., Refs. [3,4]) and increasing the mechanical strength of the membrane (e.g., Ref. [5]). To date, most studies of the effects of cholesterol on the properties of model membranes have focused on the hydrocarbon interior. Generally, in bilayers composed of lipids with saturated hydrocarbon chains, cholesterol increases lipid order due to its flat, rigid structure. In bilayers composed of lipids with *cis*-unsaturated hydrocarbon chains, cholesterol will disrupt chain packing due to its inability to pack well around the bend of the double bond [6]. These effects are especially pronounced in the gel phase.

Cholesterol is not completely miscible in membranes and at certain concentrations, cholesterol-rich domains will form (see Ref. [1]). A theoretical model for these phases was proposed by Ipsen et al. [7], which is based on the free energy of interactions between cholesterol, and chain-ordered and chain-disordered lipids. Experimental phase diagrams of cholesterol in dimyristolyphosphatidylcholine or DMPC [8], in dipalmitoylphosphatidylcholine or DPPC [9,10] and palmitolyoleoylphospatidylcholine or POPC membranes [11] have been determined using several physical techniques. These diagrams show that above the gel-to-liquid crystal phase transition of the lipid, the presence of cholesterol induces additional phases: a completely miscible disordered phase (L_d) occurring at low cholesterol concentrations, a mixed phase ($L_d + L_o$) at intermediate cholesterol concentrations, and an ordered phase (L_o) at high cholesterol concentrations. The shape of the phase diagram is thought to vary slightly between different lipid systems [12]. Direct detection of domains in regions where immiscibility should occur has been elusive using NMR, ESR [9] and fluorescence [13]. This latter fluorescence study set a maximum value on the size and lifetime of these domains.

There is evidence that cholesterol will alter the properties of a membrane surface: addition of an equimolar amount of cholesterol to phosphatidylcholine membranes produces pronounced head group expansion [14] and interdigitation of two opposing bilayers [15]. The miscibility of cholesterol in negatively charged bilayers is different than in zwitterionic bilayers, most likely due to a decrease in charge repulsion between the head groups [16]. Additionally, NMR studies indicate that when cholesterol is present in high concentrations, large perturbations in surface hydration occurs [17]. The addition of cholesterol to membranes with saturated hydrocarbon chains causes the emission of a fluorescence probe to shift to higher energies, indicating a reduction in the polarity of the membrane surface [18].

The effect that cholesterol will have on bilayer surfaces is intimately dependent on its position in membranes. While it had been long been assumed that cholesterol occupies membrane sites identical to lipid molecules, recent models indicate that under some circumstances, cholesterol can penetrate significantly into the aqueous phase [19], or even span the two leaflets of the bilayer [20]. The models both assume that cholesterol has the ability to change its position with changes in the physical properties of the membrane such as length, packing and temperature.

Based on these studies and many others not specifically cited, it is probable that the perturbations of membrane surfaces and hydrocarbon interiors caused by cholesterol will differ depending on the nature of hydrocarbon chains of the lipid and the lipid phase. In this study, we have used high-pressure fluorescence spectroscopy to investigate the effects of cholesterol on the surfaces of membranes composed of different types of lipids. High pressure has been previously been used by several groups to determine the effects of increased chain packing on the membrane structure and dynamics [21], as well as specific interactions between cholesterol and lipid [22,23]. Briefly, the application of pressure decreases the number of *cis*-gauche isomers (e.g., Ref. [24]) causing a decrease in the free volume of the membrane and increase in bilayer thickness. High-pressure fluorescence has been used to study the decrease in membrane fluidity due to cholesterol [25,26]. The microscopic compressibility of phosphatidylcholine bilayers is high in the planes parallel to the hydrocarbon chains, but much lower in the perpendicular planes due to the increase in bilayer thickness caused by straightening of the lipid chains [27].

High pressure has also been used to study changes in the surface properties of membranes due to increased lipid packing using fluorescent pH-sensitive probes [28]. When these probes are in solution or on

the surface of micelles, the application of pressure results in increased proton dissociation due to the large reduction in volume that occurs when water molecules electrostrict around the newly formed charged ions. Immobilizing the probes on zwitterionic phosphatidylcholine surfaces eliminates proton dissociation under pressure. Due to the large compressibility of the hydrocarbon chains, it was postulated that the underlying reason for this lack of volume change is that the surface does not expand and allow for the increased hydration that accompanies electrostriction, although other explanations, such as local changes in lipid structure and close proximity to the charges in the lipid head group, are possible. Highly electronegative head groups, such as phosphaditic acid (PA), show proton association under pressure that helps to alleviate head group repulsion. Changes in the protonation state of DECNA may thus offer some clues as to the positioning of cholesterol in membranes: an increase in proton dissociation under pressure would correspond to a more unrestricted environment, where the presence of cholesterol expands the spacing between the head groups by either interdigitating into the next leaflet or by spanning the two leaflets. In this study, we first characterize the effects of cholesterol on membrane surfaces at atmospheric pressure using DECNA, confirm this behavior with a second polarity-sensitive probe (Laurodan), and determine the corresponding changes in the hydrocarbon interior using a series of fluorescent fatty acid probes. We then use high pressure to gain clues into the position and possible repositioning of cholesterol in several membranes.

2. Materials and methods

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was from US Biochemical; Laurodan was from Molecular Probes (Eugene, OR) and DECNA was a generous gift from Dr. Gregorio Weber of the Dept. of Biochemistry at the University of Illinois. Unless indicated otherwise, all samples were in 50 mM Hepes buffer, pH 7, 0.16 M KCl. The majority of work was carried out using small, unilamellar vesicles (suvs) prepared by sonication as before [29] to work with more optically clear samples. Samples were used within 1–2 days of preparation. We determined the stability of suvs containing 0 and 20% cholesterol using sedimentation based on the method of Rebecchi et al. [30]. We prepared sucrose-loaded (at 180 mM sucrose) vesicles doped with 1% ^{14}C-lipid and sedimented the vesicles at 100,000 g for 30 min. The rationale was that instability of the vesicles would result in leakage of sucrose from the interior of the vesicles and cause them to remain in the supernatant. Over a 36-h period we found no evidence of any radioactivity in the supernatant at either 0 or 20 mol% cholesterol and all the radioactivity was found in the pellet, indicating that the vesicles are stable over this time period.

We also checked whether the behavior of the fluorescent probes that we used differed in multilamellar vs. small, unilamellar vesicles. For the anthroyloxystearic acid (AS) samples, the values of the anisotropy and pressure dependence of the anisotropy of 2-, 6- and 12-AS DOPC multilamellar vesicles were within error of those obtained for suvs for 0, 5, 10, 15 and 25% cholesterol. Both the mlv and suv data for the AS studies have been averaged together and are listed in Tables 2 and 3 (only 1 set of mlv samples were done). We also compared DECNA-containing DMPC mlvs vs. suvs. The atmospheric values of the degree of dissociation as a function of cholesterol concentration were within error of the values listed in Table 1, but we note that the error for these samples was somewhat high as noted in the legend. However, the pressure dependence of the

Table 1
The degree of DECNA dissociation (α) as a function of cholesterol concentration

mol% CHOL	DMPC[a]	DOPC	DOPA	DOPG
0	0.60	0.67	0.86	0.39
5	0.70	0.77	0.84	0.40
10	0.78	0.84	0.85	0.42
15	0.80	0.82	0.86	0.43
20	0.70	0.82	0.86	0.45

[a] Data are a composite of three independent sets of samples.
DECNA concentration was always 1 mol%.
DMPC samples displayed the highest error of ± 0.14; the others showed an error less than ± 0.05.
DMPC data were taken at 40°C; the others, at 22°C.

Table 2
Fluorescence anisotropy values of 2-AS and 12-AS in DOPC suvs

[Chol] (%)	2-AS	6-AS	12-AS
0	0.068	0.113	0.055
5	0.072	0.118	0.057
10	0.074	0.120	0.059
15	0.077	0.123	0.063
20	0.080	0.125	0.066

Data were taken at 22°C in 50-mM hepes buffer, pH 7, 0.16 M KCl.
Measurements were made against reference bilayers that did not contain probe.
The maximum error of these values is ±0.002.

degree of dissociation of both the mlv and suv samples was very similar and within the ±0.05 of ln K_{app} listed in Fig. 6.

Fluorescence measurements were taken on a time-resolved, phase-modulation ISS K2 spectrofluorometer equipped with Glan-Thompson polarizers (ISS, Champaign, IL). Pressure studies were carried out in a home-built optical cell based on the design of Paladini and Weber [31]. Samples were checked for reversibility after pressure release. The experimental error for anisotropy measurements was ±0.002 and was less than 1% for each intensity value. The error values reported in the tables and figures are calculated from averages of 3–5 different sets of samples as specified in the text.

2.1. Analysis of the dissociation state of DECNA

Data were analyzed as before [28] in terms of the relative intensities of the protonated form (emission maximum at 428 nm) over the intensity of the unprotonated form (emission maximum at 500). From this ratio, termed IR, the degree of dissociation, α, was calculated by:

$$\alpha = (IR_{obs} - IR_{OH})/(IR_{O^-} - IR_{obs}) \quad (1)$$

where IR_{obs} is the observed intensity ratio, IR_{OH} is the ratio of the completely protonated species, determined from the emission spectrum in SDS micelles at pH 7, and IR_{O^-} is the ratio of the completely deprotonated form, determined in CTAB micelles at pH 13. The direction of the reaction is taken to be towards dissociation; thus, $K = [H^+][DECNA^-]/[HDECNA]$. Since our studies were conducted in buffered solution and hence constant proton concentration,

$$K_{app} = [DECNA^-]/[HDECNA] = \alpha/(1-\alpha). \quad (2)$$

We present our results in terms of the change in ln K_{app} with pressure and note that the apparent volume change, $\Delta V_{app} = -d(RT \ln K_{app})/dp$.

In the data presented, we measured the atmospheric value of α six times by placing the sample in a cuvette and determining the emission spectrum. The pressure dependence of α was measured 3–4 times by placing the samples in the inner cuvette of the pressure cell and inserting the cell in the optical module of the fluorometer, and measuring the emission spectra as a function of pressure. We found that in the pressure cell, the error of the initial atmospheric value of α was always high due to small differences in the positioning of the pressure cell in the light path. However, the pressure dependence of α was always the same and the sample-to-sample error was low. For this reason, the atmospheric values in Fig. 3 have a much higher error than the values at elevated pressure as noted. The only sample set, where the discrepancy between the atmospheric value of α measured in the cuvette differed from the value obtained in the pressure cell, was the 10% cholesterol samples, whose average in the pressure cell gave a slightly lower value. In Fig. 3, we have used the more accurate atmospheric α value measured in a cuvette as taken from Table 1. The pressure values have been scaled to this atmospheric value.

Table 3
DOPC microscopic compressibilities (per kbar)

[Chol] (%)	2-AS	6-AS	12-AS
0	−0.070	−0.086	−0.073
5	−0.077	−0.098	−0.083
10	−0.087	−0.100	–
15	−0.090	−0.104	−0.091
25	−0.090	−0.101	−0.079

Since the fluorescence lifetime of the three probes vary slightly, for comparison, the atmospheric values of the anisotropy were scaled to $\tau = 11.8$ ns using a ratio of the equation $A/A_0 - 1 = RT\tau/\eta V$, where A_0 is the anisotropy in the absence of rotational motion and equal to 0.312, T is the temperature, R is the gas constant, V is the rotational volume and η is the viscosity (for details, see Scarlata, 1991).
Values of the compressibilities have an error of ±0.004.

2.2. Assessment of the microscopic compressibility

The microscopic compressibility was calculated as before [27] through the observed fluorescence anisotropy A, which can be related to the average cosine squared of the angle (θ) swept out by the fluorophore during its lifetime:

$$\langle \cos^2\theta \rangle = \left[1 + 2\langle A \rangle / A_0\right]/3. \quad (3)$$

This angle represents a volume element whose behavior with pressure (p) can be used to determine the isothermal compressibility β through a free volume model. Since

$\beta = -1/V(\mathrm{d}V/\mathrm{d}p)$, then

$$V(p) = V(\mathrm{atm}) \times (1 - \beta p). \quad (4)$$

A complete discussion of this method can be found elsewhere [27].

3. Results

3.1. Studies at atmospheric pressure

As a prelude to the pressure studies, we characterized the changes in the properties of model membranes induced by cholesterol using fluorescent probes. The first of these was DECNA (Fig. 1). This probe has a hydrocarbon tail attached to a pH-sensitive fluorescent head group. At pH 7, room temperature and atmospheric pressure, the probe is almost completely dissociated when embedded in uncharged micelles (OG) or when its short-chained analog (6-propanoylnaphthol) is free in solution. When placed in negatively charged SDS micelles, the probe is completely protonated. Placing the DECNA into DOPC bilayers, where the zwitterionic head group is electrically neutral results in decreased proton dissociation in a manner similar to fatty acids whose pK shift to neutral upon membrane incorporation (see Ref. [28]). The values for the degree of dissociation (α), as calculated by Eq. (1), for several cholesterol concentrations in various lipid membranes are given in Table 1. Although the error is somewhat high, it appears that in DMPC the presence of cholesterol may initially cause an increase in the dissociation state of DECNA, which possibly reverses at 20% cholesterol. In DOPC and DOPG, a more continuous increase is observed.

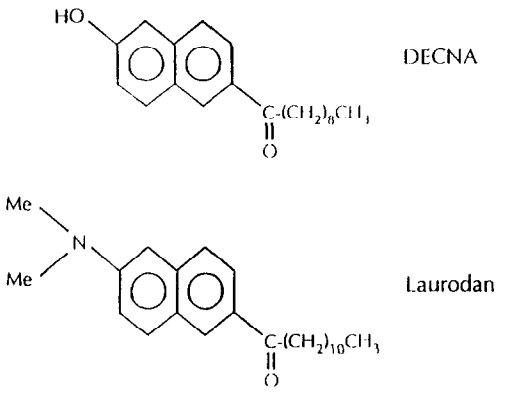

Fig. 1. Structures of two of the probes used in this study.

Changes in bilayer surface caused by cholesterol were confirmed using a second probe Laurodan (see structure in Fig. 1). Laurodan was incorporated into DMPC bilayers at 40°C, and DOPC and DOPG bilayers at room temperature, and the shift in the center of spectral mass was measured as a function of cholesterol concentration. The emission energy of the fluorescent head group of Laurodan is very sensitive to changes in environmental polarity [32], local solvation [33], lipid phase [18] and cholesterol [19]. Consistent with this later report, we find that the addition of cholesterol shifts the emission to higher energies in all three types of lipid, although the trends are somewhat different (Fig. 2). Comparing the behavior of Laurodan and DECNA with cholesterol in the different sets of lipids (Fig. 2 and Table 1), we find that the trends observed for the two probes are similar but not identical. Although a detailed analysis of the Laurodan data was not done, it is apparent that cholesterol alters the surface of vesicles composed of varying lipid molecules.

The above data indicate that cholesterol alters the lipid surface with some possible differences between saturated and unsaturated lipids. To better understand the nature of these differences, we assessed the changes that cholesterol induces in the hydrocarbon interior of DOPC and DMPC bilayers using a series of three fatty acid probes: 2-, 6- and 12-Anthroyloxystearic acid (AS). Based on studies by Thulborn and Sawyer [34], the fluorescent anthroyloxy side chain is positioned at progressively deeper depths in the 2-, 6- and 12-AS series. The fluorescence

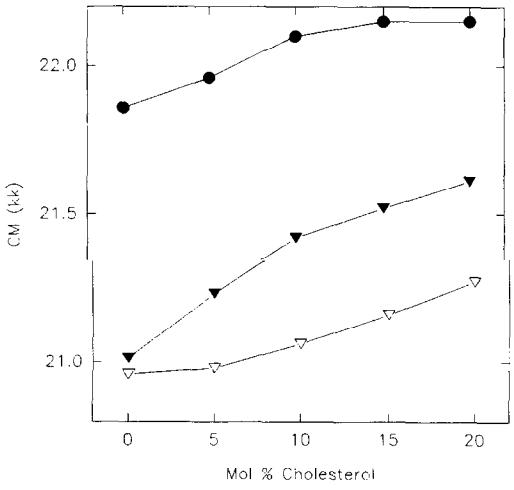

Fig. 2. Change in the center of spectral mass of Laurodan as a function of cholesterol concentration in (●) DMPC suvs at 40°C, and (▽) DOPC and (▽) DOPG suvs at 22°C.

anisotropies of these probes showed systematic increases with cholesterol from 0 to 20 mol% in both DMPC at 40°C and DOPC at room temperature with little change in lifetime in accord with previous measurements [35]. Values for DOPC are listed in Table 2. These data show that the anisotropy of 2- and 12-AS increase with cholesterol concentration to the same extent (11% for 2-AS and 13% for 12-AS). The anisotropy values of 6-AS, on the other hand, appear to show less sensitivity to cholesterol. However, this apparent reduced sensitivity is due to the higher anisotropy values obtained for this probe, which is probably due to the positioning of the anthroyl moiety around the *cis* double bond of the oleyl chains. If we relate the anisotropy values to the precession angle swept out by the probe during its lifetime (see Eq. (3)), we find that in going from 0 to 20 mol% cholesterol, 2-AS decreases from 46.22 to 44.75°; 6-AS decreases from 40.70 to 39.20°, and 12-AS decreases from 47.82 to 46.47°. Thus, the decrease in the precession angle for the three probes are similar, indicating that cholesterol is producing isotropic decreases in the free volume of the hydrocarbon region of the bilayer. In DMPC bilayers, the values of the anisotropy and precession angle of both 2- and 12-AS increased with cholesterol content to the same extent, again indicating isotropic decreases in free volume in the hydrocarbon region.

3.2. Effects of cholesterol on the pressure behavior of DECNA and AS probes

As mentioned, we have found that when the head group of DECNA is an 'unrestricted' environment such as a micelle or free in solution, the application of pressure will cause proton dissociation with a large reduction of volume due to electrostriction of water around the charged groups ($\Delta V = -5.2$ ml/mol). On the other hand, in PC bilayers, electrostriction does not occur [28]. If cholesterol acts to increase the distance between lipid head groups, then its presence should allow for increased DECNA dissociation under pressure. We tested this hypothesis in several different lipid systems.

3.2.1. DOPC bilayers

The emission spectrum of DECNA in zwitterionic, unsaturated lipid DOPC (dioleoyl–PC) with varying amounts of incorporated cholesterol was monitored as a function of pressure. In the absence of cholesterol, the protonation state of DECNA is not affected by pressure. The presence of 5 or 10% cholesterol promoted dissociation up to 0.8 and 1.0 kbar, respectively, after which the equilibrium remained relatively constant (Fig. 3). 20% cholesterol produced no significant overall change in DECNA dissociation. For all cholesterol concentrations, the data are fairly linear up to ~ 800 bar. Therefore, we can determine the significance of these changes by comparing the error in the least-square slope of the three sets of data for each cholesterol concentration (we note that *t*-tests with Bonferroni corrections showed statistical differences between the 0 mol% and 5 and 10 mol% data sets). The slopes obtained are: 0.005 ± 0.004 for 0%; 0.22 ± 0.10 for 5%; 0.21 ± 0.05 for 10% and 0.07 ± 0.04 for 20%. Thus, the difference in slopes between the 0% and 5 and 10% samples, and between the 5%, 10% and 20% samples, is greater than the error of the slopes. The volume changes calculated from these slopes are: -0.12 ± 0.10 ml/mol for 0%; -5.2 ± 2.3 ml/mol for the 5%; -5.0 ± 1.2 ml/mol for 10%; and -1.67 ± 1.0 for 20%. These data show that the volume changes for proton dissociation of DECNA in the 5 and 10 mol% cholesterol sample are close to the value obtained when the probe is free in solution

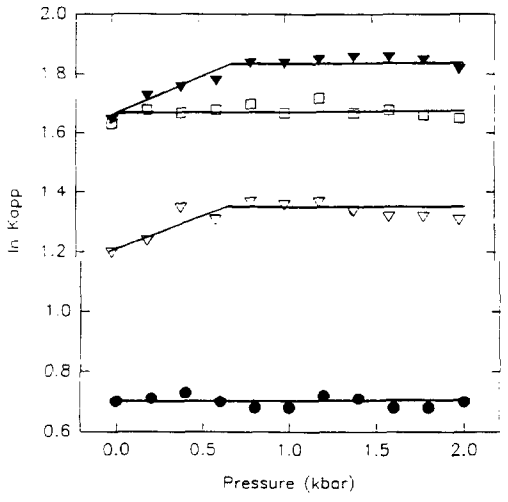

Fig. 3. DECNA dissociation as a function of pressure in DOPC suvs at 22°C. Dissociation is in terms of ln K_{app} (see Section 2). The points presented are an average from data of three samples. The error at atmospheric pressure is ±0.1, while the maximum error of the values at elevated pressure is ±0.05 (see Section 2). Cholesterol concentrations are: (●) 0, (▽) 5%, (▽) 10% and (□) 20%.

(−5.5 ml/mol). The ability of DECNA to dissociate at 5 and 10% indicates that cholesterol is causing the DECNA environment to become more unrestricted, either by allowing for increased hydration, or by changes in the local environment around the probe. However, it appears that only at a certain level, these changes can be accommodated, and after this particular pressure is reached, no further dissociation occurs. From Fig. 3, it is apparent that the effects of cholesterol are not systematic (see Section 4).

The increase in DECNA dissociation with pressure observed at 5 and 10 mol% cholesterol is reversed at 20 mol%. This reversal could be due to the sequestering of cholesterol into domains, or a shift in its average position in the membrane. To determine which mechanism is most probable, we again employed the anthroyloxy fatty acid probes and monitored the change in anisotropy as a function of pressure. The intensity, which is proportional to lifetime, remained unchanged under pressure. From these data we computed a rotational volume from which we extracted a local or microscopic compressibility (β) using Eqs. (3) and (4). We reasoned that if cholesterol sequestered into domains under pressure, then the anthroyloxy probes would be transferred from a cholesterol-rich to a cholesterol-depleted environment, and this would be reflected in a reversal of the compressibility to values seen at lower cholesterol concentrations. Alternately, if cholesterol changed its average position in the membrane, then the relative compressibilities of 2 − and 12-AS would differ, since we have previously found that the microscopic compressibility of DOPC bilayers is constant with membrane depth [27].

We measured the change in anisotropy and intensity with pressure of the AS probes in DOPC-cholesterol membranes at room temperature, and related these to the compressibility. Experimental data for one of these probes is displayed in Fig. 4. The calculated compressibility values are listed in Table 3. Initial studies were done in 50 mM Hepes–0.16 M KCl buffer at pH 7. Neither the anisotropies or compressibilities were altered when the buffer system was changed to MES, pH 5 or Tris, pH 8 indicating that our results are unaffected by the protonation state of stearic acid. The compressibility values of the samples without cholesterol in accord with the macroscopic values reported by Braganza and Worcester [35] using diffraction techniques.

The data in Table 3 show that the compressibilities of 2-AS labeled membranes decrease until 15% cholesterol and level at 25%. Likewise, the compressibilities of the 6-AS labeled membranes de-

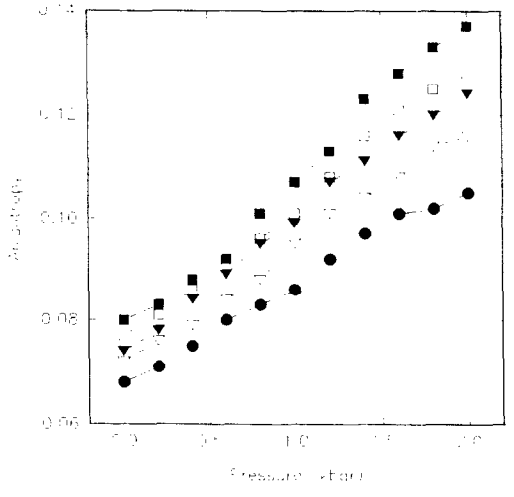

Fig. 4. Increase in the fluorescence anisotropy of 2-AS in DOPC suvs at pH 8 and 22°C as a function of pressure at (●) 0%, (▽) 5%, (▽) 10%, (□) 15% and (■) 25% cholesterol. Each point has an error of ±0.002.

crease up to 15% cholesterol, and possibility increase at 25% cholesterol. The 12-AS labeled membranes also decrease up to 15% but show a pronounced reversal at 25%. The decrease in compressibility with increasing cholesterol content is in accord with the trend seen in the modulus of compressibility as determined by Needham and Nunn [5] using uniaxial compression. A decrease in compressibility with cholesterol content is also in accord with the high pressure studies of Braganza and Worcester [35], whose diffraction studies indicated this trend, and is in accord with microscopic compressibility values extracted from the polarization–pressure data using diphenylhexatriene [25].

The data in Table 3 show a differential response of the three probes to cholesterol at higher cholesterol concentrations. If cholesterol was sequestered into cholesterol-rich domains at higher concentrations, then we would expect that all of the probes would show reversal. It is possible that if cholesterol domains form, they may not be extensive enough to change the properties of the surrounding lipids. Alternately, the reversal of 12-AS as opposed to the two other less buried probes may indicate a shift in the average location of cholesterol to positions similar to the lipid chains (see Section 4).

3.2.2. DOPA studies

In our previous work, we found that when DECNA was embedded in DOPA bilayers, whose head groups are highly electronegative, it tended to protonate under pressure, presumably to alleviate the increased charge repulsion that occurs with increased lipid packing [28]. If cholesterol acted to expand the surface, then DECNA would be in a more unrestricted environment, and cholesterol should diminish the DECNA protonation under pressure. This idea was tested by examining the effect of cholesterol on DECNA dissociation in DOPA suvs under pressure (Fig. 5). At 0% we observed protonation under pressure. At 5 and 10% cholesterol, we find that the tendency for DECNA to become protonated decreases from atmospheric pressure to 0.8–1.0 kbar and at higher pressures, protonation sharply increases, indicating either phase separation or phase change. This behavior is analogous to DOPC. The slopes obtained for the three sets of data at each cholesterol concentration in the 0 to 1 kbar range

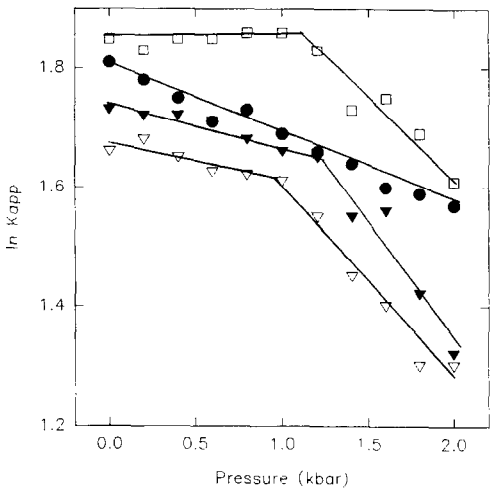

Fig. 5. DECNA dissociation as a function of pressure in DOPA suvs at 22°C. Dissociation is in terms of ln K_{app}. The data are a composite of three sets of samples with a maximum error of the values at elevated pressure is ±0.05. Cholesterol concentrations are: (●) 0%, (▽) 5%, (▽) 10% and (□) 20%.

are: -0.19 ± 0.006 for 0%; -0.068 ± 0.013 for 5%; -0.065 ± 0.15 for 10%; and $+0.020 \pm 0.011$ for 20%. The corresponding volume changes are: $+4.5 \pm 0.14$ ml/mol for 0%; $+1.6 \pm 0.30$ ml/mol for 5%; $+1.5 \pm 37$ ml/mol for 10% and -0.4 ± 0.3 ml/mol for 20%. We note that the volume change of the 0 mol% sample is greater than that previously determined ($+2.5$ ml/mol with an estimated error of ± 1.0 ml/mol). This difference may be attributed to differences in sample preparation, spectroscopic equipment and sampling ranges, pressure cells and possibly inclusion of points greater than $\alpha = 0.9$. Nevertheless, the trend in the behavior of DECNA in these samples as opposed to DOPC and DMPC are the same.

If changes in DECNA behavior due to cholesterol were due primarily to increased lipid packing, then increasing the lipid packing by lowering the temperature should give similar results. We compared the pressure behavior of DECNA in DOPA-suvs containing 15 and 25% cholesterol at 22 and 14°C (data not shown). We found that decreasing the temperature caused the dissociation of DECNA at atmospheric pressure to decrease, and also causes the pressure behavior to be similar to that of lower cholesterol concentrations.

3.2.3. DOPG studies

We previously found that under pressure, DECNA will, unexpectedly, deprotonate in negatively charged DOPG bilayers [28]. Although other mechanisms are possible, we postulate that the underlying reason for this dissociation is either the larger DOPG head groups can accommodate increased hydration with DECNA dissociation due to its two hydroxyl groups, or these groups may better hydrate the anionic form of the probe. In an attempt to better understand this anomalous pressure behavior, we measured the change in DECNA dissociation behavior when cholesterol is incorporated into DOPG bilayers. We find that the presence of cholesterol causes the pressure behavior of DECNA to revert to a volume change of approximately zero (data not shown). If the PG head groups could initially accommodate an increase in DECNA hydration under pressure, and if cholesterol acts to expand the surface, then we would expect more pronounced dissociation of DECNA under pressure. The observation that cholesterol reverses DECNA dissociation under pressure indicates that the anomalous behavior of DECNA in PG membranes is due to specific interactions between the PG groups and DECNA that become disrupted as cholesterol is incorporated into the membrane.

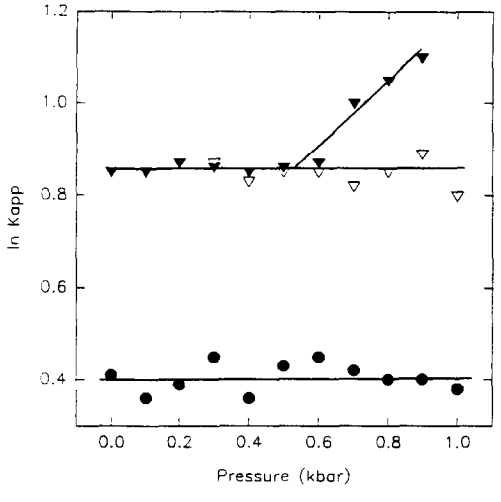

Fig. 6. DECNA dissociation as a function of pressure in DMPC suvs at 40°C. Dissociation is in terms of ln K_{app} with an error at elevated pressure of ±0.05. Cholesterol concentrations are: (●) 0%, (▽) 5% and (▽) 20%.

3.2.4. DMPC studies

We tested the behavior of DECNA in a lipid system with saturated hydrocarbon chains, DMPC. The experiments were conducted at 40°C where the lipid is in the fluid phase at atmospheric pressure and undergoes transition to the gel phase at 1 kbar [21]. The results are shown in Fig. 6. Interestingly, we find that the application of 600 bar pressure does not affect the dissociation state of DECNA even in the presence of 20% cholesterol, but at higher pressures and a transition becomes visible for the 20% cholesterol sample. [1]

4. Discussion

Many cellular processes involve the association of membrane species to the bilayer surface. Because factors that influence these associations may influence other cellular events, it is important to understand how association depends on the properties of the membrane surface. In this study we have focused on the effect that the prominent membrane component cholesterol has on the properties of membrane surfaces. In the three lipid systems studied (DOPC, a zwitterionic, unsaturated lipid; DMPC, a zwitterionic saturated lipid; and DOPG, an unsaturated negatively charged lipid), the presence of cholesterol causes the emission energy of a polarity sensitive probe, Laurodan, to shift to higher energies. Our spectral data (not shown) indicated that this shift results from an apparent increase in a higher energy population at the expense of a lower energy population. This behavior could be interpreted in molecular terms such as a loss of the double hydrogen-bonded probe population and a gain in the singly hydrogen-bonded population [33], or a decrease in the ability of the solvent to relax [36], or a destabilization of the emission dipole [32]. However, we prefer to simply interpret this blue shift as being due to a dilution of

[1] We note that attempts to determine the changes in compressibility using AS probes were unsuccessful because the values of the compressibility increased steadily with increasing pressure. This behavior is seen with many liquids, and it precludes us from assigning a unique compressibility for each sample. We note that cholesterol increases the compressibility of these bilayers.

the ionic species in the head groups surrounding the probe by neutral uncharged cholesterol molecules. Whichever mechanism is operative, it is apparent that the presence of cholesterol decreases the polarity of bilayer surfaces, and that this decrease does not appear to be specific.

Changes in membrane surface properties caused by cholesterol were also examined using the fluorescent pH indicator DECNA. When its short-chained probe analog is free in solution, or when DECNA is embedded in neutral, octylglucoside micelles at pH 7, DECNA is ~ 85% dissociated. On the other hand, in PC bilayers, the dissociation state of DECNA decreases to ~ 67%. This decrease in pK upon membrane incorporation is typical of fatty acids. In the highly electronegative environment of DOPA, the hydroxyl proton of DECNA becomes delocalized on the PA surface, causing the dissociation of DECNA to reach 85% [28]. In the three lipid systems examined (DMPC, DOPC and DOPG), incorporation of ~ 5–15% cholesterol increased DECNA dissociation. DECNA is 85% dissociated in DOPA bilayers, and cholesterol did not affect the dissociation state of DECNA in these bilayers. Taken together, changes in the dissociation state of DECNA with cholesterol could be interpreted as being due to an increase in the spacing between lipid head groups, thereby allowing the dissociation state to come closer to its value in more unrestricted environments. While this behavior may also be explained by a change in the average position of the probe with respect to the phosphate groups, we would not expect this change to be very similar for the two types of probes and in the different types of lipids.

It is possible that other molecular changes besides head group expansion can be invoked to explain the changes in the ionization of DECNA. These involve cholesterol-induced perturbations of the probe environment stemming from repositioning of the probe or lipid, so that the probe environment more closely resembles water. These changes would then cause the polarity around the probe to increase. Although changes in local polarity are difficult to assess by DECNA emission, these changes are straightforward by Laurodan emission. Assuming the similarly-structured Laurodan mimics DECNA, then the emission shift to higher energies of Laurodan is not consistent with cholesterol-induced repositioning of the probe or lipid, and support the idea of head group expansion.

The most previous cholesterol studies have been carried out using multilamellar vesicles, while the majority of our studies used sonicated vesicles. As detailed in Section 2, identical data were obtained using either multilamellar or unilamellar vesicles. Several years ago, Huang and Mason [37] established that in suvs, 67% of the lipid molecules are located in the outer leaflet. While the area per head group is much greater in the outer leaflet vs. the inner, the weighted average of the two leaflets is approximately equal to the area of lipids on planar surfaces. We assumed that the probes are randomly located in the membrane based on the ability of fatty acids and detergents to flip-flop between two leaflets, and based on the high rate of lateral diffusion of these molecules in the disordered phase below 30% cholesterol [7]. However, it is possible that the probes are not randomly distributed in the two membrane leaflets. Noting that our measurements view an average of probe emission in an ensemble of membrane positions, and that the head group spacing in the inner and outer leaflets of suvs differs [37], if the probes were not evenly distributed, then the results obtained for mlvs would differ from those obtained for suvs. Although we do not believe that our results are complicated by varying solubilities of the probes in different lipid domains, since preferential solubilities would not be expected to give behavior indicative of head group expansion. We also note that the lifetimes of the putative cholesterol domains are sufficiently short with respect to the time scale of our measurements; therefore, we will only be viewing bulk effects [13]. However, we cannot discount the possibility that the size and leaflet distribution in the suvs change as the cholesterol content increases. It is important to note that because the motions of the membrane components are dynamic as compared to the time scale of the measurements, only the average of the membrane perturbations caused by cholesterol are observed.

Numerous studies have recently suggested that the presence of cholesterol alters the phase diagram of lipids [7,9,10,12,20]. In addition to the disordered or fluid (L_d) phase where the acyl chain have conformational freedom, and the gel or solid phase (S) where the chains are locked into lattice positions for

long times, cholesterol induces an ordered phase (L_o) where the acyl chains are extended and orientationally ordered, but in contrast to the S phase, can diffuse in the plane parallel to the membrane and rotate freely along the axis perpendicular to the membrane plane. There also exists a region in the phase diagram, where both the disordered phases ($L_d + L_o$) coexist over a wide range of temperatures.

The position of cholesterol in these phases is still under debate. Our DECNA studies support the notion that the presence of cholesterol results in an expansion of the membrane surface. Expansion of the membrane surface would be expected if the membrane position of cholesterol is such that only the hydroxyl group protrudes into the head group region, and the rings reside in the hydrocarbon region. Our data is also consistent with cholesterol occupying hydrocarbon positions that are diagonal or partially perpendicular to the hydrocarbon chains. However, this latter position is not consistent with the increase in anisotropy observed for the 12-AS probe located in deeper regions of the membrane. Our data are consistent with the model of Sankaram and Thompson [20] that suggests that cholesterol can interdigitate into the next leaflet or span the two leaflets of the membrane. These authors supported their model by measurements, showing that the effective chain length of the membranes studied (DMPC, DPPC and DSPC with varying amounts of cholesterol) were lower than that of cholesterol. Cholesterol is thought to span the two leaflets in the disordered phase and interdigitate in the ordered phase. While spanning of the two leaflets would be expected to increase the area between the lipid head groups, interdigitation in the ordered phase may not. From the DMPC phase diagram of Almeida et al. [8], at 40°C and atmospheric pressure, the bilayers will be in the disordered phase up to $\sim 12.5 \pm 3$ mol% cholesterol and in the mixed $L_d + L_o$ up to $\sim 28\%$. Both the Laurodan and DECNA results can be reconciled in terms of an initial expansion from 5–10% followed by a leveling, and possibly even a partial reversal, as cholesterol molecules occupy positions between the lipid chains. Although the phase diagram of DOPC has not yet been published, that of POPC has already been [11]. The 0 and 5 mol% at atmospheric pressure appear to correspond to surface expansion produced by cholesterol in the disordered phase and the phase diagram of POPC shows the mixed phase starting at 10 mol% cholesterol and 22°C, whereas our DOPC data correspond more to the disordered phase at this concentration, and the mixed phase occurring thereafter.

Our data support the idea that the phase diagram of cholesterol in a particular lipid determines the position of cholesterol in a membrane, and that this position, in turn, determines whether cholesterol will increase the area between the lipid head groups. Thus, a shift in the conditions, where cholesterol expands the surface to one where the surface is condensed, is subject to the same factors that modulate the particular phase. We further characterized the effect of cholesterol on membrane surfaces by the pressure behavior of DECNA. It has been previously shown that DECNA will tend to dissociate under pressure due to the large volume decrease that accompanies hydration around the newly formed ions (electrostriction) with $\Delta V = -5.2$ ml/mol [28]. When DECNA is in the restrictive environment of a membrane surface, then its dissociation state is unaffected by pressure. The presence of 5 mol% cholesterol in DOPC bilayers allowed for increased DECNA dissociation when the pressure was initially increased. Raising the concentration to 10% cholesterol increased both the extent of DECNA dissociation and pressure at which the increase in dissociation ceased. At 20 mol% cholesterol, the dissociation state is close to its value in an unrestricted environment, and no further dissociation occurs.

The idea that cholesterol is expanding the surface is supported by the DOPA studies where, in the absence of cholesterol, the surface becomes protonated, but in the presence of cholesterol, pressure-induced protonation is greatly diminished in the first 1000 bars. Conditions where this apparent expansion should be diminished, such as lower temperature or much higher pressure, also support this argument. Support also comes from DOPG studies showing that cholesterol reverses the tendency for the anomalous increase in dissociation under pressure by decreasing the specific DECNA-head group interactions that give rise to this behavior. The presence of cholesterol decreases the surface density and reverses the particular effects of the head groups on DECNA.

Although the behavior of DECNA in unsaturated

lipids is consistent with the idea that cholesterol increases the spacing between the lipid head groups, this does not seem to be the case for DECNA in DMPC bilayers. Under conditions where the membranes should be in the pure L_d phase (5 mol% cholesterol) and in the mixed, $L_d + L_o$ phase (10, 15 and 20 mol%), pressure-induced dissociation of DECNA is inhibited. Thus, in this saturated lipid, it is possible that expansion of cholesterol produced in DMPC bilayers is not enough to allow DECNA dissociation due to electrostriction. Inhibition of DECNA dissociation can also be understood in terms of the increase in surface packing that occurs when cholesterol begins to occupy sites identical to the lipid chains. At high cholesterol concentrations and high pressure (i.e., 20 mol% and 600 bars), our data suggest a transition from the mixed phase to the ordered phase, where the dissociation state of DECNA is higher.

For unsaturated lipids, it is not clear whether the cessation of increased DECNA dissociation at higher pressure corresponds to the onset of the mixed phase region, or simply to the increased packing of the lipid head groups. If we assume that in the L_d phase the addition of cholesterol increases the area between the lipid head groups and, in turn, allows for proton dissociation of DECNA under pressure, but in the mixed phase this affect is reversed, then the pressure at which reversal occurs may be related to the onset of the mixed phase. However, our data show that this is not the case. Also arguing against this idea are the data collected for pressure behavior of anthroyloxyl-stearic acid probes located in the hydrocarbon region of DOPC. Relating the rotational motion of the 2-, 6- and 12-AS probes to a free volume, and obtaining a microscopic compressibility ($\beta = 1/V(dV/dp)$) show that throughout the pressure range used here, a constant β was obtained. This result argues against phase separation of cholesterol under pressure. The data in Table 3 indicate a leveling of the change in compressibility at 25 mol% cholesterol for 2- and 6-AS, and a reversal for 12-AS. Although other explanations are possible, we interpret this result as being due to a repositioning of cholesterol to positions identical to the lipid chains.

In summary, our results from these pressure and atmospheric studies show that changes in different types of model membranes caused by cholesterol, as detected by low amounts of fluorescent probes, can be readily analyzed in terms of the theoretical phase diagrams of cholesterol–lipid mixtures [7,9,10,19,20]. Specifically, our data support the membrane positions of cholesterol as proposed by Sankaram and Thompson [10]. From these studies, it is apparent that the phase of the cholesterol–lipid mixture, which is a function of the concentration, temperature, nature of lipid and pressure, directly modulates an expansion of the lipid surface produced by cholesterol, which in turn could influence the binding of membrane-associating species.

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