Elastic deformation and failure of lipid bilayer membranes containing cholesterol

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ABSTRACT Giant bilayer vesicles were reconstituted from several lipids and lipid/cholesterol (CHOL) mixtures: stearoyloleoylphosphatidylcholine (SOPC), bovine sphingomyelin (BSM), diarachidonylphosphatidylcholine (DAPC), SOPC/CHOL, BSM/CHOL, DAPC/CHOL, and extracted red blood cell (RBC) lipids with native cholesterol. Single-walled vesicles were manipulated by micropipette suction and several membrane material properties were determined. The properties measured were the elastic area compressibility modulus K, the critical areal strain α_c, and the tensile strength τ_{lys}, from which the failure energy or membrane toughness T_f was calculated. The elastic area expansion moduli for these lipid and lipid/cholesterol bilayers ranged from 57 dyn/cm for DAPC to 1,734 dyn/cm for BSM/CHOL. The SOPC/CHOL series and RBC lipids had intermediate values. The results indicated that the presence of cholesterol is the single most influential factor in increasing bilayer cohesion, but only for lipids where both chains are saturated, or mono- or diunsaturated. Multiple unsaturation in both lipid chains inhibits the condensing effect of cholesterol in bilayers. The SOPC/CHOL system was studied in more detail. The area expansion modulus showed a nonlinear increase with increasing cholesterol concentration up to a constant plateau, indicating a saturation limit for cholesterol in the bilayer phase of ~55 mol% CHOL. The membrane compressibility was modeled by a property-averaging composite theory involving two bilayer components, namely, uncomplexed lipid and a lipid/cholesterol complex of stoichiometry 1/1.22. The area expansion modulus of this molecular composite membrane was evaluated by a combination of the expansion moduli of each component scaled by their area fractions in the bilayer. Bilayer toughness, which is the energy stored in the bilayer at failure, showed a maximum value at ~40 mol% CHOL. This breakdown energy was found to be only a fraction of the available thermal energy, implying that many molecules (~50–100) may be involved in forming the defect structure that leads to failure. The area expansion modulus of extracted RBC lipids with native cholesterol was compared with recent measurements of intact RBC membrane compressibility. The natural membrane was also modeled as a simple composite made up of a compressible lipid/cholesterol matrix containing relatively incompressible transmembrane proteins. It appears that the interaction of incompressible proteins with surrounding lipid confers enhanced compressibility on the composite structure.

INTRODUCTION

Cholesterol (CHOL) is present in varying amounts in the plasma and organelle membranes of cells, and much is known about its interactions with different lipids (Demel et al., 1972a; Demel and De Kruyff, 1976; Yeagle et al., 1977; Yeagle, 1985, 1987). This interaction ranges from a strong association with certain lipids such as sphingomyelin (Demel et al., 1977; Barenholz and Thompson, 1980) to an apparently weak or nonexistent interaction with lipids that contain multiple double bonds in both acyl chains (Demel et al., 1972b; Guyer and Bloch, 1983). One central feature is that cholesterol strongly affects the upper portions of lipid acyl chains, leaving them less able to change conformations, while the center of the bilayer becomes more disordered (McIntosh, 1978; Scott and Kalaskar, 1989). This interaction results in an increase in membrane cohesion, as shown by increases in mechanical

stiffness of membranes and decreases in membrane permeability to water, and remains to be fully quantified (Evans and Needham, 1987; Needham et al., 1988; Fettiplace and Haydon, 1980, for review).

Cholesterol also affects the temperature-dependent phase behavior of artificial membranes made from single lipid species to the extent that at ~50 mol% cholesterol the anomolous specific heat associated with the gel to liquid crystalline phase transition is completely abolished (Ladbrooke et al., 1968; Estep et al., 1978; Maybrey et al., 1978; Needham et al., 1988). Attempts have been made to interpret the temperature-dependent phase behavior of lipid/cholesterol systems in terms of the occurrence of specific stoichiometric complexes and by thermodynamic mixing models (Engelman and Rothman, 1972; Presti et al., 1982; Ipsen et al., 1987, 1989; Needham et al., 1988). We should also be able to extend these models to explain the cohesive properties of the lipid/cholesterol membrane.

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The maximum amount of cholesterol that is incorporated into reconstituted lipid bilayers is widely assumed to be ~50 mol% because at this concentration the enthalpy change associated with the gel-to-liquid crystalline phase transition becomes nonexistent. Other estimates for the cholesterol solubility limit have been determined. Small angle neutron scattering on artificial membranes of dimyristoylphosphatidylcholine-cholesterol gave a solubility limit of ~45 mol% CHOL (Knoll et al., 1985) and x-ray studies on egg phosphatidylcholine-cholesterol indicate the limit to be ~50 mol% CHOL (Lecuyer and Dervichian, 1969). Some natural membranes, however, in particular in the lens of the eye, can contain higher cholesterol concentrations, ~78 mol% CHOL (Li et al., 1985). The solubility limit for cholesterol in liquid phase lipid membranes may therefore show a subtle dependence on lipid molecule structure.

The composition and structure of the red blood cell and other natural membranes have been extensively studied, but the contributions to membrane cohesive properties by the various components are poorly characterized. Fatty acid analysis of natural red blood cell membranes shows that some lipid acyl chains can contain up to six double bonds and that ~17% of the total fatty acid contains four to six double bonds (Cooper, 1970). Thus, in membranes like the red blood cell, these unsaturated lipids make up only a small portion of the total lipid. In other nonplasma membranes, such as the disk membranes in retinal rod outer segments, polyunsaturated lipids with at least six double bonds per lipid molecule are relatively more abundant (Drenthe et al., 1980). Hence, another aspect of bilayer cohesion that is of particular interest is its dependence on lipid unsaturation (the number of double bonds per acyl chain) and the influence of cholesterol on these lipids. Experiments on monolayers have shown that the area per molecule increases with the number of double bonds in the hydrocarbon chains of the lipid (Demel et al., 1972b). Lipid molecules in which multiple double bonds were confined to only one chain showed a reduction in mean molecular area with cholesterol. However, the condensing effect of cholesterol was virtually eliminated when the lipid contained more than two cis double bonds per chain. These measurements were carried out at a monolayer surface pressure of 12 dyn/cm, which is widely recognized to be much lower than surface pressures in bilayers (Evans and Waugh, 1977). It is therefore of interest to examine these highly unsaturated systems as bilayers.

Thus, although much is known about lipid/cholesterol bilayer systems, several interesting aspects such as mechanical properties and stability, lipid:cholesterol stoichiometry, and maximum bilayer cholesterol content remain to be fully investigated. From a material science stand point, lipid membranes can be described in terms of area

expansion modulus, tensile strength, critical areal strain at failure, and membrane toughness or strain energy. Membrane area compressibility represents the resistance of the membrane to isotropic area dilation and is characterized by an elastic area expansion modulus K; tensile strength is given by the membrane tension at lysis (rupture) τ_{lys}; the critical areal strain is the fractional increase in membrane area at failure α_c; membrane toughness T_f represents the work done on the membrane up to failure. These parameters can be measured for natural cell and reconstituted vesicle membranes by a micromanipulation technique. We have improved the micropipette technique and have carried out a systematic study involving three distinct types of lipid (stearoyloleoylphosphatidylcholine, SOPC; bovine sphingomyelin, BSM; and diarachidonylphosphatidylcholine, DAPC) in combination with cholesterol up to and including saturating levels. Also, in an attempt to dissect the value for the intact red blood cell (RBC) membrane compressibility modulus into the contributions made by lipid and, by inference, transmembrane proteins, we extracted the native lipids and cholesterol from a sample of RBCs and reconstituted them as lipid bilayer vesicles.

EXPERIMENTAL METHODS

Extraction of RBC lipids

The RBC lipid was extracted according to a modified procedure of Dr. M. Rossi at UCSF (personal communication). Outdated blood was obtained from the Red Cross and 250 ml of AB+ blood was centrifuged at 1,310 g for 5 min. The serum and white blood cells, etc. were aspirated and discarded. The RBCs were washed three times with 0.85% Fisher Celline (Fisher Scientific Co., Pittsburgh, PA) isotonic saline solution. The yield was 100 ml of packed cells. To 100 ml of packed RBCs, 550 ml of cold isopropanol containing 10^{-4} M BHA (2(3)-t-butyl-4-hydroxyanisole; Sigma Chemical Co., St. Louis, MO) as antioxidant was added in a 1-liter Erlenmeyer flask. It was mixed with a glass rod that was flattened at the end. The flask was left in a fume hood for 1 h at room temperature. 350 ml of cold chloroform was added to the flask, which was then sealed with a ground glass stopper and parafilm. The flask was refrigerated overnight at 4°C. The resulting precipitate was removed by vacuum filtration through a #1 filter (Whatman Inc., Clifton, NJ) in a pyrex millipore funnel. The yield was 780 ml of filtrate. To 780 ml of filtrate, 156 ml of 0.05 M KCl was added. The mixture was centrifuged in corex test tubes at 1,310 g for 10 min. Two clear layers were visible and the top layer was aspirated and discarded. The bottom layer contained the RBC lipid extract in chloroform. Nitrogen was blown into the extract for 15 min in a 500-ml round-bottom flask, which was then stoppered and sealed with parafilm and stored at 17°C.

The phospholipid concentration of our extract was determined using the phosphorous assay of Bartlett (1959). 5 ml of RBC extract was dried under nitrogen and resuspended in 8 ml of 2:1 chloroform/methanol. 2-ml aliquots were assayed in S/P Dispo tubes (phosphate-free) and flash-dried at 150°C. Phosphorous standards (KH_2PO_4 stock solution) of the following concentrations were used for comparison with the lipid extract: 0.05, 0.075, 0.100, 0.125, 0.150, and 0.200 mM. 0.5 ml of 10 N H_2SO_4 was added to all the dried aliquots and aqueous standards. Each tube was vortexed and left overnight in a rack in an oven at 150–160°C.

The next morning the tubes were removed from the oven and allowed to cool for 5 min. Two drops of H_2O_2 were added to each tube and the tubes were returned to the oven for 2 h. After removal and cooling, 4.6 ml of 0.22 wt/wt% ammonium molybdate solution and 0.2 ml Fisk-Subbarrow reagent were added to each tube, vortexed, and covered with a glass stopper. The tubes were placed in boiling water for 12 min, and then cooled to room temperature. All tubes were vortexed and analyzed for phosphorous content using a Milton Roy 1201 spectrophotometer at 830 nm.

The presence of cholesterol in the extracted RBC lipids was qualitatively detected by the Salkowski test (sterol in chloroform plus an equal volume of H_2SO_4 yields a red-purple color). The concentration of cholesterol in the RBC extract was determined by a colorimetric assay (Boehringer Mannheim GmbH, Mannheim, FRG). Briefly, the enzyme, cholesterol oxidase, oxidizes cholesterol to cholestenone and H_2O_2. The peroxide reacts with methanol in the presence of catalase to give formaldehyde. In the presence of ammonium ions, the formaldehyde reacts with acetylacetone to give a yellow lutidine dye. Five samples of RBC lipid extract, 10 ml each, were dried under nitrogen. 1 ml of 4:1 isopropanol/chloroform was added to each vial. 25 ml of this mixture from each of the five samples was tested by the enzymatic assay as described above. The absorbance was measured by a spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) at 405 nm. The standards included cholesterol in the following concentrations: 0, 5, 10, 20, and 25 mg/ml.

Vesicle preparation and micromanipulation

The preparation of giant vesicles from lipid solutions and the procedures for carrying out thermomechanical studies are well documented in several recent publications concerning the micromanipulation of such structures (Kwok and Evans,1981; Needham et al., 1988; Needham and Evans, 1988; Needham, 1990). Briefly, lipid and lipid/cholesterol mixtures were made up in a chloroform/methanol solution. It was found necessary to sonicate the freshly prepared lipid cholesterol solutions to ensure complete mixing of lipid and cholesterol before vesicle formation. 20–40-μm-diam vesicles were formed by gentle rehydration of dried lipid lamellae from a teflon substrate using sucrose solutions (160 mOsm).

The sucrose-containing vesicle sample was diluted with 160 mOsm glucose and then resuspended in an equal volume of hyperosmotic glucose (180 mOsm) containing 0.2 g% albumin. This ~170-mOsm solution containing a more dilute suspension of vesicles was then added to the manipulation chamber. Although few in number, large unilamellar vesicles were identified for the aspiration test. The slight deflation of the vesicles produced a small projection in the suction pipette as shown in Fig. 1. After a prestress, the pipette suction pressure was increased using an in-line syringe from a nominally low (1,000 dyn/cm^2) holding pressure in increments up to a subfailure level, followed by incremental decreases in suction pressure to return to the holding pressure. Finally, the suction pressure was again increased in increments to produce vesicle lysis (failure). The corresponding change in vesicle projection length in the pipette was recorded on video. Micropipette suction pressure was measured via an in-line pressure transducer.

Subsequent analysis of pipette and vesicle geometry allowed the calculation of the relative membrane area change α (vesicle area change, ΔA, divided by an initial, low stress area, A_o) in a response to induced membrane tension τ due to vesicle pressurization (Kwok and Evans, 1981). The experiments were carried out in a temperature-controlled chamber at 15°C. At this temperature, changes in osmolarity due to evaporation losses, and concomitant decreases in vesicle volume, were negligible during a modulus measurement. Also, the presence of trapped solutes prevented any volume changes due to water filtration. Thus, changes in projection length in the pipette were solely due to changes in isotropic membrane tension and not volume changes. The elastic area expansion modulus was determined from the slope of plots of membrane isotropic tension vs. membrane area change, for membrane tensions greater than ~0.5 dyn/cm, as shown in Fig. 2. The vesicle membrane lysis tension τ_{lys} was simply determined from the measured tension when a given vesicle ruptured, and was rapidly aspirated up the pipette. For lipid systems that contained large amounts of cholesterol,
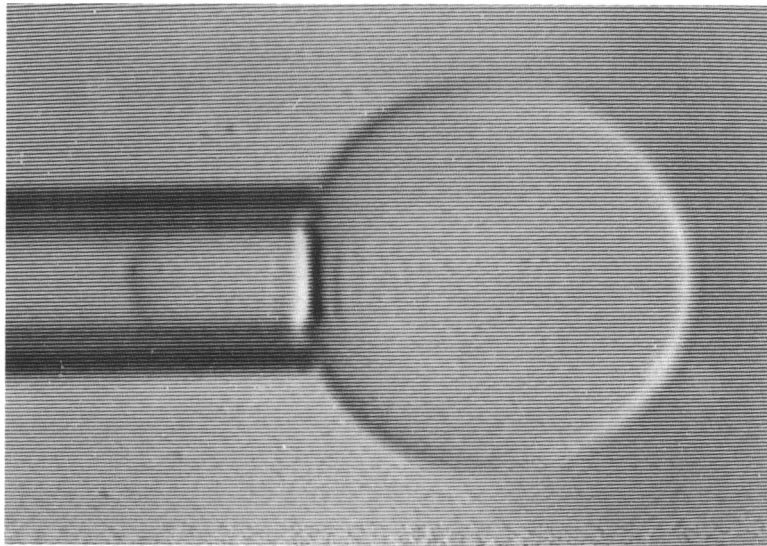

FIGURE 1   Videomicrograph of a giant lipid bilayer vesicle (20 μm diam) aspirated by micropipette (8 μm diam). The length of the vesicle projection in the pipette is a sensitive measure of the total vesicle membrane area change. The applied pipette suction pressure together with the pipette and vesicle geometry give the induced isotropic membrane tension.
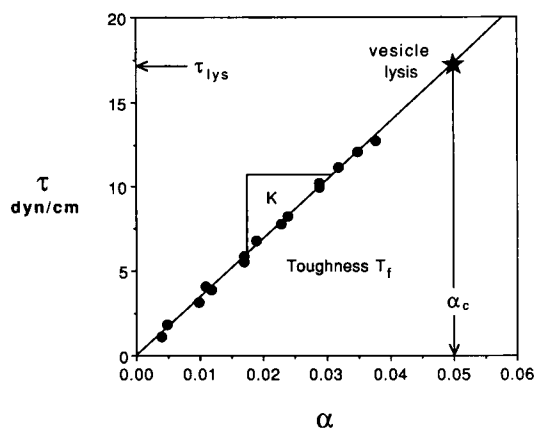
FIGURE 2 Stress vs. strain plot for micropipette pressurization of a lipid vesicle (SOPC/CHOL 62/38). Vesicle membrane tension τ resulting from the applied micropipette suction pressure is plotted against the areal strain α (i.e., the observed increase in vesicle membrane area ΔA relative to an initial low stressed state A_o). The slope of the τ vs. α plot is the elastic area compressibility modulus K. The star represents the point of membrane failure at the critical areal strain α_c and tensile strength τ_{lys}. The area under the plot represents the energy at membrane failure, i.e., the membrane toughness T_f.

lysis occurred at high suction pressures ($>$60,000 dyn/cm^2) that were above the range of the pressure transducer installed in the apparatus. In these cases, although an estimate of the critical pressure was obtained from a calibrated suction syringe, the actual tension at lysis was determined from an extrapolation of the τ vs. α plot to the measured critical area at failure α_c.

In view of the problems associated with pipette charging (Katnik and Waugh, 1990), the pipette and the microchamber were grounded. This, together with a precoating of the pipette and glass chamber surfaces with albumin and the presence of albumin in the measuring chamber, greatly reduced the incidence of premature vesicle lysis that is apparently caused by static charge build-up on the glass surfaces.

Several lipid and lipid/cholesterol systems were studied. The lipids were synthetic SOPC (18:0/18:1), BSM (12:0/18:0, i.e., based on stearoyl), and DAPC (20:4/20:4) obtained from Avanti Polar Lipids, Inc., Birmingham, AL. The cholesterol-containing membranes included the RBC lipid extract containing ~40 mol% native cholesterol, and the following nominal lipid/cholesterol mixtures: SOPC/CHOL, 0–89 mol% CHOL; DAPC/CHOL, 0, 50, and 80 mol% CHOL; and BSM/CHOL, 50 and 80 mol% CHOL. These lipid and cholesterol mixtures represent the nominal stoichiometric concentrations of lipid and cholesterol in the initial organic solvent solutions from which the vesicles were made. It is recognized that the vesicles reconstituted from RBC lipids form symmetric bilayers, unlike in natural RBC membranes, where the four major classes of lipids are asymmetrically distributed (Schwartz et al., 1984).

RESULTS

Chemical analysis

Table 1 shows the results of our chemical analysis of the RBC lipid extract where we determined cholesterol content and total phosphorous to obtain the concentration of

TABLE 1 Chemical analysis of the red blood cell lipid extract

Component	Assay	Concentration
		mmol/10 ml final extract
Phosphorous	Bartlett	7.08
Cholesterol	Boehringer-Mannheim	4.74

The cholesterol concentration in the extracted lipid sample was therefore found to be 4.74 × 100/(7.08 + 4.74) = 40.1 mol% CHOL with respect to phospholipid.

phospholipids as mmol/10 ml final extract solution. The cholesterol in this particular sample of RBCs was determined to be 40.1 mol% with respect to the phospholipid. The analysis does not include minor components such as monoglycerides, triglycerides, and free fatty acids, and so represents an upper limit based on total phospholipid.

Stress vs. strain

The measured values for the elastic area expansion modulus, the critical areal strain at failure, and the membrane tension at failure are presented in Table 2 for all lipid systems studied. A typical stress vs. strain plot is shown in Fig. 2. Vesicle membrane tension τ produced by applied micropipette suction is plotted against the relative increase in vesicle membrane area α. Increasing membrane tension caused a linear increase in vesicle membrane area and showed a perfectly reversible (elastic) recovery upon decreasing membrane tension. The slope of this line $\Delta\tau/\Delta\alpha$ is the elastic area expansion modulus K. Subsequent increases in membrane tension produced failure of the membrane and lysis of the vesicle at some critical areal strain and critical tension, giving α_c and τ_{lys} ($\tau_{lys} = K\alpha_c$).

We excluded from our analysis any response of the membrane to applied tensions less than ~0.5 dyn/cm because in this range applied tensile stresses go primarily into smoothing out thermal undulations (Evans and Rawicz, 1990). The critical areal strain due to isotropic area dilation of the membrane was therefore calculated from a linear regression fit to the data points that were obtained for membrane tensions in excess of this low threshold value.

For all systems in which the tension at lysis could be accurately measured, via the in-line pressure transducer, and for the remainder, in which a calibrated syringe gave an estimate of the applied suction pressure, membrane expansion was linearly proportional to the applied membrane tension and no ductility up to the point of failure was observed. The area under the stress-strain plot represents the amount of work per unit area that was stored in the vesicle membrane up to the point of failure

TABLE 2   Cohesive properties of vesicle bilayer membranes

Lipid system	mol%	K dyn/cm	α_c	τ_{lys} dyn/cm	T_f J/mol	$A_{m(calc)}$ Å²
SOPC	0	193 ± 20	0.030 ± 0.004	5.7 ± 0.2	34 ± 9	65.0
	14	216 ± 12	0.041 ± 0.009	8.7 ± 1.8	65 ± 29	59.1
	28	244 ± 24	0.051 ± 0.012	12.6 ± 2.7	106 ± 46	53.2
	38	333 ± 9	0.051 ± 0.009	16.9 ± 3.2	132 ± 52	49.0
	43	609 ± 44	0.031 ± 0.004	18.4 ± 2.9	79 ± 13	46.9
	43	568 ± 36	0.031 ± 0.002	17.8 ± 1.8	86 ± 20	46.9
	48	710 ± 48	0.029 ± 0.005	20.6 ± 3.7	83 ± 30	44.8
	50	781 ± 45	0.025 ± 0.004	19.7 ± 3.2	64 ± 25	44.0
	53	907 ± 73	0.023 ± 0.002	19.4 ± 2.2	57 ± 9	42.7
	58	1,207 ± 135	0.022 ± 0.002	24.9 ± 2.1	72 ± 10	41.9
	63	1,251 ± 167	0.025 ± 0.006	30.2 ± 6.0	68 ± 7	41.9
	63	1,165 ± 68	0.025 ± 0.005	28.1 ± 5.7	91 ± 33	41.9
	78	1,286 ± 105	0.022 ± 0.003	28.0 ± 2.2	78 ± 13	41.9
	78	1,222 ± 150	0.021 ± 0.002	24.7 ± 3.9	65 ± 17	41.9
	89	1,190 ± 62	0.026 ± 0.003	30.9 ± 3.7	102 ± 22	41.9
BSM	50	1,718 ± 484	0.016 ± 0.004	23.2 ± 5.9	49 ± 14	40
	80	1,732 ± 354	0.013 ± 0.003	22.3 ± 3.5	34 ± 6	40
DAPC	0	57 ± 14	0.036 ± 0.014	2.3 ± 0.6	19 ± 11	80
	50	102 ± 24	0.043 ± 0.010	4.5 ± 0.6	32 ± 10	58.5
	80	67 ± 13	0.048 ± 0.012	3.4 ± 1.7	27 ± 11	58.5
RBC extract	40	423 ± 41				
	80	783 ± 75				

Material property parameters for lipid vesicle bilayer systems at 15°C composed of a variety of lipid types and lipid/cholesterol compositions. Shown are the elastic area compressibility modulus K, the critical areal strain α_c, the tensile strength τ_{lys}, and the toughness T_f. The last column gives the calculated average area per molecule $A_{m(calc)}$ for each bilayer system and composition according to our stoichiometric model.

and is given by $K(\alpha_c)^2/2$. This can be converted into an energy/mole by multiplying by the area per mole (area per molecule × Avogadro's number) derived for each lipid and lipid/cholesterol system from experimental measurements and from a simple mixing model that is outlined in the following Theoretical Analyses section.

Elastic area expansion modulus

The elastic area expansion modulus for all systems studied vs. nominal mol% cholesterol is plotted in Fig. 3. It was not possible to make a separate chemical analysis for each individual vesicle tested, so the results are presented in terms of nominal mol% cholesterol. The SOPC/CHOL series showed a nonlinear increase in expansion modulus with increasing addition of cholesterol up to a constant plateau of ~1,210 dyn/cm that began at a cholesterol concentration between 53 and 58 mol%. This maximum value for the saturated SOPC/CHOL system represents an increase of approximately six times compared with that of the pure SOPC bilayer. Even though a large excess of cholesterol was added to the initial lipid/cholesterol solution from which the vesicles were made, no further change in vesicle membrane expansion modulus was observed for the higher nominal concentrations up to 89 mol% CHOL. Several systems were in fact

repeated to check for reproducibility. As discussed in the Theoretical Analyses section, this may be taken to indicate a maximum value for the amount of cholesterol that is incorporated in vesicle bilayers made from these solu-
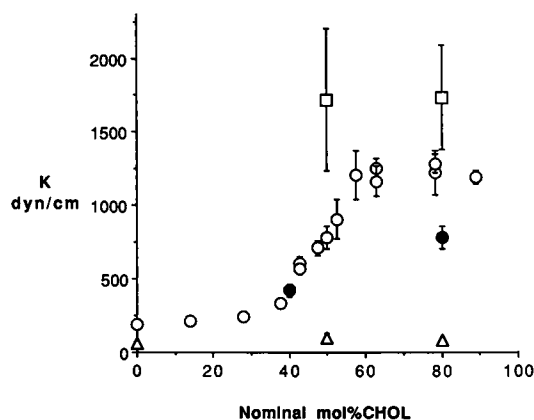

FIGURE 3   Elastic area compressibility modulus K for all lipid and lipid/cholesterol systems studied is plotted vs. the nominal mol% cholesterol in the chloroform/methanol solution from which the vesicles were made. Open circles are SOPC/CHOL; open squares are BSM/CHOL; open triangles are DAPC/CHOL; filled circles are RBC lipids/CHOL. All measurements were carried out at 15°C.

tions. Our improved resolution in the modulus measurement also showed a small jump in modulus between 40 and 45 mol% CHOL. This small discontinuity indicates a possible structural transition that may be detectable by molecular spectroscopic techniques.

The sphingomyelin/cholesterol system was found to have a much higher modulus than SOPC/CHOL and appeared to contain a maximum amount of cholesterol for the nominal cholesterol concentrations of 50 and 80 mol%. Since the phase transition of pure BSM is ~40°C, a single component BSM vesicle preparation with the lipid in the liquid crystalline phase could not be studied at our working temperature of 15°C.

In contrast to the enhanced cohesion conferred on SOPC and BSM lipids, the mixing of cholesterol with the highly compressible DAPC lipid showed only a modest increase in expansion modulus, by less than a factor of 2. Vesicles made from RBC lipid extract containing 40 mol% native cholesterol showed an expansion modulus that was similar to the SOPC/CHOL system at the same cholesterol content. In line with the other systems, the addition of excess cholesterol to the RBC lipid extract (to give a nominal cholesterol concentration of 80 mol% CHOL), caused an increase in elastic modulus.

Critical area strain

Fig. 4 shows how the critical areal strain α_c at membrane failure varied with nominal mol% CHOL for the SOPC/CHOL system. Curiously, the areal strain at vesicle lysis showed an initial increase, peaked at ~30–40 mol% CHOL, and then sharply decreased between 40 and 45 mol% CHOL with no further change after a nominal concentration of 58 mol% CHOL. Critical areal strains for the other systems are given in Table 2. The α_c values
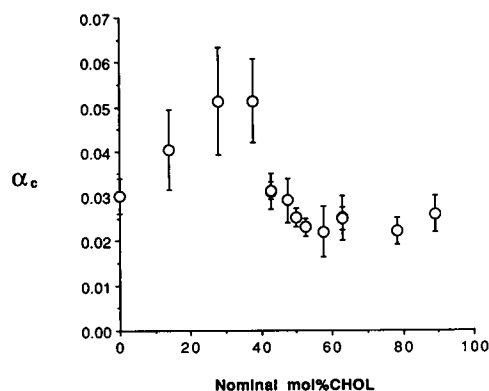
for BSM/CHOL were smallest at 0.013–0.016, while DAPC and DPAC/CHOL membranes showed area expansions of 0.036–0.048 before lysis.

Lysis tension

The membrane tension required to rupture the vesicle membranes is given in Table 2 for all systems and plotted for the SOPC/CHOL system vs. nominal mol% CHOL in Fig. 5. Once again, a saturation limit was observed for this property. For the SOPC/CHOL bilayers, an increase in cholesterol concentration caused a steady increase in tensile strength and turned into a plateau region (at 25–30 dyn/cm) when the nominal mol% CHOL was above ~58 mol% CHOL. The tensile strength of BSM/CHOL membranes was fairly high, ~22–23 dyn/cm, while, consistent with their low moduli, the DAPC/CHOL were relatively weak with tensile strengths between 2.3 and 4.5 dyn/cm.

Critical energy at failure

The area under each stress vs. strain plot (Fig. 2) represents the elastic energy stored in the vesicle membrane per unit area when it broke and is shown in Fig. 6, plotted against the nominal mol% CHOL for the SOPC/CHOL system. It is simply calculated from the area of the enclosed triangle in Fig. 2 in terms of the two most reliably measured parameters K and α_c, and is given by $K(\alpha_c)^2/2$. This failure energy also showed a maximum value around 40 mol% CHOL and an almost constant value above 58 mol% CHOL. The failure energies for the other synthetic lipid systems at a given cholesterol concentration were less than the respective SOPC/CHOL systems as listed in Table 2.
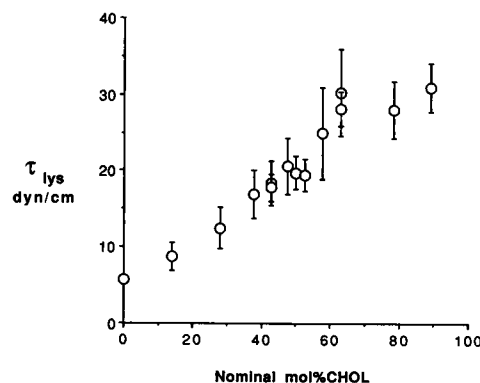
FIGURE 4 Critical areal strain α_c vs. nominal mol% CHOL for the SOPC/CHOL system.

FIGURE 5 Membrane tension at vesicle lysis τ_{lys} vs. nominal mol% CHOL for the SOPC/CHOL system.
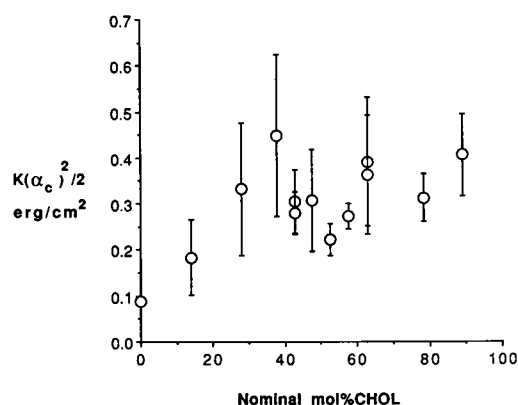
FIGURE 6 Energy per unit area ta failure $K(\alpha_c)^2/2$, stored in the bilayer membrane due to elastic deformation vs. nominal mol% CHOL for the SOPC/CHOL system.

THEORETICAL ANALYSES

Elastic area expansion modulus for SOPC/cholesterol membranes

By analogy with common macroscopic composite theory for fiber- or particulate-reinforced materials (in which a matrix material is stiffened by the incorporation of particles of a higher modulus material), a simple two-dimensional molecular composite model was developed for the lipid/cholesterol membrane in order to interpret the nonlinear elastic modulus data for the SOPC/CHOL system. In this approach we identify two components that make up the bilayer and assume that, during isotropic loading of the composite, the stresses in the particulate and matrix components are equal and continuous across the boundaries between the components, and that the total strain is a linear combination of the strains of each component multiplied by their respective area fractions.

It is not possible, from our approach, to distinguish whether cholesterol rapidly exchanges between lipids (so that each lipid molecule is affected to a certain extent), or whether the cholesterol forms a "permanent" complex with a certain fraction of lipids in the membrane. We take the view that cholesterol forms an association with the lipid such that, on the average, two components effectively make up the bilayer. We identify the two components as uncomplexed lipid (UL) and a "tight" lipid/cholesterol complex (L/C). Such a simple division is consistent with our earlier interpretation of SOPC/CHOL thermal area transition data (Needham et al., 1988) and is analogous to the thermodynamic models of Ipsen et al. (1987, 1989) who identify two conformational states of the lipid molecules, i.e., an ordered state due to

the interaction of cholesterol with lipid causing a straightening of the lipid acyl chains (the lipid:cholesterol complex) and a conformationally disordered state in which the chains retain their rotameric defects (uncomplexed lipid). Fig. 3 shows that the area modulus increases from a value of 193 dyn/cm for the single component SOPC bilayer to a plateau value of $\sim$1,210 dyn/cm with increasing mol% cholesterol.

Several phases can be identified in the lipid/cholesterol/water system, including: the aqueous solution containing sucrose/glucose as dissolved solutes and low concentrations (10^{-10} M) of lipid and cholesterol; a monolayer of lipid and cholesterol at the air-aqueous interface; the bilayer phase, made up of lipid and cholesterol (uncomplexed lipid and lipid:cholesterol complex); and nonbilayer, cholesterol crystallites. In our experiments we sample directly with the micropipette only the bilayer vesicle phase of the multiphase system. The limiting value shown in Fig. 3 of between 53 and 58 mol% CHOL may be taken to indicate that at nominal concentrations of cholesterol greater than this no more cholesterol is incorporated in the bilayer and that at this point of cholesterol saturation the bilayer is again composed of a single species, namely the lipid/cholesterol complex. Based on the area modulus behavior we postulate the following: at nominal cholesterol concentrations above the modulus limit, the bilayer vesicle phase is fully saturated with cholesterol and excess cholesterol is present in the aqueous phase as cholesterol crystallites; at nominal cholesterol concentrations below this limit the bilayer vesicle phase contains cholesterol at the nominal concentration.

Therefore, given the postulate that the bilayer consists of two components, a lipid:cholesterol complex and an uncomplexed lipid, we can write an equation for the composite bilayer in which the area expansion modulus of the membrane K_m is a linear combination of the moduli of the two components K_{UL} and $K_{L/C}$ scaled by their area fractions a_{UL} and $a_{L/C}$:

$$K_m = \left\{ \frac{a_{UL}}{K_{UL}} + \frac{a_{L/C}}{K_{L/C}} \right\}^{-1}. \tag{1}$$

The modulus of each component is given by the measured moduli for pure SOPC bilayers at 0 mol% CHOL (193 dyn/cm), and for the lipid:cholesterol complex at the saturation limit (1,210 dyn/cm). To model the behavior we chose a best fit to the data using a saturation limit of 55 mol% CHOL, i.e., a limiting stoichiometry of 1/1.22 for the SOPC/CHOL system.

The area fractions of uncomplexed lipid a_{UL} and lipid:cholesterol complex $a_{L/C}$ can be formulated in terms of the nominal mol% CHOL X_C and the areas for the uncomplexed lipid A_{UL} and the lipid:cholesterol complex

$A_{L/C}$, for a lipid/cholesterol complex of stoichiometry 1/1.22.

$$a_{UL} = \frac{A_{UL}\left\{(100 - X_c) - \left(\frac{X_c}{1.22}\right)\right\}}{A_{UL}\left\{(100 - X_c) - \left(\frac{X_c}{1.22}\right)\right\} + A_{L/C}\left(\frac{X_c}{1.22}\right)}. \quad (2a)$$

Similarly, the area fraction of the lipid/cholesterol complex is given by

$$a_{L/C} = \frac{A_{L/C}\left(\frac{X_c}{1.22}\right)}{A_{UL}\left\{(100 - X_c) - \left(\frac{X_c}{1.22}\right)\right\} + A_{L/C}\left(\frac{X_c}{1.22}\right)}. \quad (2b)$$

Since the area per SOPC molecule has not yet been experimentally determined, we approximate the area per molecule for the uncomplexed lipid A_{UL} in excess water from that measured for egg lecithin by Lecuyer and Dervichian (1969), i.e., 65 A^2. The same study also showed that cholesterol has a gradual condensing effect on the molecules of lecithin leading to an approximate limiting value of 48 A^2 for lipid in the cholesterol-saturated bilayer. We therefore take this value to be the area per lipid in the lipid:cholesterol complex.

For cholesterol, experiments involving spread monolayers lead to a limiting area per molecule of 37 A^2 and, in line with Lecuyer and Dervichian (1969), we assume that in the bilayer phase the cholesterol molecules occupy a constant area of 37 A^2 whatever the mole fraction. These values then lead to the following areas per molecule A_{UL} and $A_{L/C}$ for uncomplexed lipid and the lipid/cholesterol complex of stoichiometry 1/1.22:

$$A_{UL} = 65 \text{ A}^2$$

$$A_{L/C} = 48 \text{ A}^2 + 1.22(37 \text{ A}^2) = 93\text{A}^2.$$

When these values are substituted in Eqs. 2a and 2b and these results are substituted in Eq. 1 together with the limiting elastic moduli, a theoretical value for the modulus is obtained for the simple composite membrane as a function of mol% CHOL in the bilayer phase. This relation is plotted in Fig. 7 and shows a nonlinear increase in bilayer area expansion modulus with increasing mol% CHOL. Thus, a limiting value for cholesterol saturation in the bilayer phase (and by inference, the stoichiometric ratio of the lipid/cholesterol complex) of ~55 mol% CHOL models the experimental data remarkably well and corresponds to a stoichiometry of 1/1.22 for the lipid/cholesterol complex.

DISCUSSION

Stress vs. strain

For a single bilayer vesicle aspirated by a micropipette as shown in Fig. 1, an increase in suction pressure produces
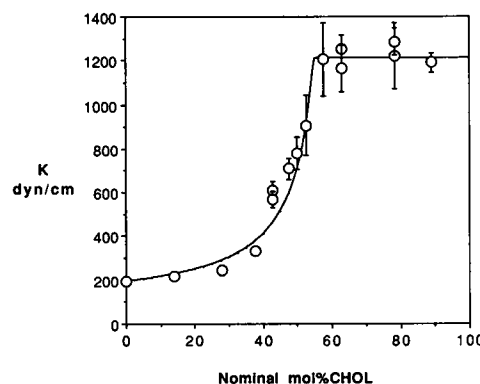

FIGURE 7   Comparison between experimentally determined elastic area expansion modulus K (*open circles*) and the theoretical values (*solid line*) derived from the molecular composite model for the SOPC/CHOL bilayer system. The area expansion modulus of the composite membrane K_m is a combination of the area expansion moduli of the two components, namely, uncomplexed lipid and lipid/cholesterol complex, and their respective area factors ($a_{UL}, a_{L/C}$) in the membrane according to the equation $K_m = \{a_{UL}/K_{UL} + a_{L/C}/K_{L/C}\}^{-1}$. The theoretical curve represents a stoichiometry for the lipid/cholesterol complex of 1/1.22, corresponding to a limiting saturation of 55 mol% CHOL.

an increase in the projection length in the pipette. By considering the pipette and vesicle geometry, the induced membrane tension and the concomitant vesicle area change can be readily calculated to give a stress vs. strain relation up to the point of membrane failure, as shown in Fig. 2. The reversible, linear relation from tensions greater than ~0.5 dyn/cm all the way up to failure demonstrates a perfectly elastic material behavior that is reminiscent of brittle failure in that the membrane shows no ductility and all the energy is stored in elastic deformation.

Elastic area expansion modulus

In this study we considered lipid systems that represent certain basic features of natural and artificial membranes. Vesicle membranes made from phosphatidylcholines and in particular SOPC are widely recognized as behaving to a first approximation like many natural membranes, even though natural membranes are composed of several lipid types containing acyl chains of various degrees of unsaturation. This broad generalization is supported in our study by the observation (Fig. 3) that the extracted RBC lipids, including native cholesterol at 40 mol%, were found to have an expansion modulus that was essentially the same as that measured for the SOPC/CHOL system at the same cholesterol concentration.

The other lipid systems were chosen to represent certain extremes. Sphingomyelin is found together with

cholesterol in many natural membranes and appears to have a strong association with the cholesterol molecule (Barenholz and Thompson, 1980). On the other hand, the monolayer experiments of Demel et al. (1972b) indicated that the highly unsaturated DAPC lipid showed virtually no condensing effect with cholesterol, at least at the relatively low surface pressure of 12 dyn/cm.

Our measurements on bilayers support the inference that sphingomyelin and cholesterol form a very cohesive membrane since the area modulus (~1,725 dyn/cm) is the highest yet measured for any membrane system. Also, unlike the SOPC/CHOL system, BSM/CHOL bilayers appear to be saturated with cholesterol at 50 mol% CHOL, since at the higher nominal cholesterol concentration of 80 mol% CHOL the modulus was the same as at a 50 mol% CHOL. The difference in gel–liquid crystalline phase transition temperatures T_m between SOPC (278°K) and BSM (313°K) would strictly preclude a direct comparison; i.e., measurements for BSM/CHOL membranes were made at a temperature below the gel–liquid crystalline transition of the pure lipid (normalized temperature $[T/T_m - 1]$ of -0.08), while those for SOPC/CHOL were made above T_m (at $[T/T_m - 1] = +0.036$). Comparative measurements that might be made on other systems such as DPPC/CHOL ($T_{m\,DPPC} = 315°K$) and over a range of temperatures may resolve the tempeature contribution to the area modulus for cholesterol-containing system.

In sharp contrast to the BSM/CHOL, SOPC, and SOPC/CHOL systems, DAPC bilayers were found to be highly compressible and showed a reduced interaction with cholesterol. It is clear that the presence of multiple double bonds in both chains can effectively weaken the lipid bilayer, both with and without cholesterol. The DAPC bilayers were much more compressible and much more fragile than other, common, more saturated lipids. We have seen this effect before for an extremely fragile (unpolymerized) butadiene lipid preparation that had an apparent area modulus of 46 dyn/cm and a lysis tension of 0.7 dyn/cm (Evans and Needham, 1987; Needham, Sackmann; and Evans, unpublished results). In line with the monolayer studies of Demel et al. (1972b), cholesterol did not have the same condensing effect on bilayers made from the highly unsaturated DAPC lipids as it did on lipids of low unsaturation, like SOPC. Only a marginal increase in area modulus was obtained for the nominal 1/1 DAPC/CHOL system and further excesses of cholesterol did not produce any increase in modulus. Again, a temperature effect may make some contribution since, although it has not been measured, T_m for DAPC must be very low, ~233°K (16:0/22:6 phosphatidylcholine is reported to have a T_m of -30 to $-25°C$, from fluorescence polarization studies [Sklar et al., 1979]), and so our

modulus measurements were made at the relatively high normalized temperature of $+0.215$.

Turning now to the molecular composite model for the SOPC/CHOL bilayer, Fig. 7 shows that a simple property-averaging theory can account for the nonlinear increase in area modulus that is observed when the mol% of cholesterol in the bilayer is increased. In the model, no assumptions were made regarding size or shape of component domains. Whether homogeneously mixed or separated into component-rich domains, the correlation with a macroscopic composite theory indicates that each molecular component brings to the bilayer phase "its characteristic modulus." It is only required that the isotropic stress is uniform throughout the membrane and that the nominal lipid/cholesterol concentration is representative of the lipid/cholesterol ratio in the bilayer phase. We did make some attempt to ensure that the lipid and cholesterol molecules were well mixed by sonicating the chloroform/methanol solution before solvent drying and vesicle rehydration. In the absence of a composition assay for each single vesicle tested, we noted that the modulus measurements did increase slightly for the sonicated preparations as compared to the freshly made, unsonicated solutions. This indicated that any small influence of kinetic mixing restrictions were in fact reduced if not eliminated. Also, the relatively small error bars indicate a population of vesicles of homogeneous composition. The change in area modulus demonstrates that cholesterol is included in the bilayer and illustrates the extent of its interaction with a given lipid. It gives the actual point of saturation for cholesterol inclusion and, by inference, indicates that the stoichiometry of the purported lipid/cholesterol complex is 1/1.22.

Membrane failure: critical areal strain, tensile strength, and failure energy

Fig. 4 shows the form of the changes in critical areal strain with increasing mol% of cholesterol. The fact that the critical areal strain increases then decreases demonstrated that bilayer failure (at least for two components) cannot be ubiquitously linked to a single value for critical strain as was postulated earlier (Evans and Needham, 1987). Nor can it be due to the attainment of a single critical tension (Fig. 5), or to the storage of a constant amount of elastic energy per unit area (Fig. 6). All these properties did, however, show some constancy above ~58 mol% CHOL, which, along with the modulus measurements, indicated a constant bilayer composition for nominal cholesterol concentrations >58 mol% CHOL.

The peak in critical areal strain and the steadily increasing tensile strengh are combined (as $K(\alpha_c)^2/2$) to show that the lipid membrane is toughened by the

addition of cholesterol, but only up to a certain point, around 40 mol% CHOL. This may be coincidence, but it is interesting to note that the natural RBC membrane also contains ~40 mol% CHOL. BSM/CHOL membranes showed the highest area expansion modulus, but because they could only be subjected to a small areal strain the overall energy stored at failure was somewhat less than for the SOPC/CHOL membranes. The DAPC and DAPC/CHOL membranes showed a relatively high areal strain but their low expansion modulus again limited the amount of stored energy at failure.

This analysis can be taken a step further by converting the failure energy per unit area $K(\alpha_c)^2/2$ into an energy per mole or membrane toughness T_f (Table 2) by multiplying by Avogadro's number and the appropriate area per molecule. Membrane toughness for the SOPC/CHOL system is shown in Fig. 8. The maximum toughness at 40 mol% CHOL and the relatively constant values at cholesterol concentrations above 58 mol% CHOL are retained since the calculated area per molecule, derived from our stoichiometric model and shown in Table 2, decreases linearly with increasing mol% CHOL, from 65 to 41.7 A^2. These area per molecule changes are in close agreement with x-ray studies on egg PC (Lecuyer and Dervichian, 1969) and monolayer studies on other phosphatidylcholines (Demel et al, 1972b). The amount of energy that is stored in the SOPC/CHOL membrane when it breaks ranges from 35 J/mol for the single component SOPC membrane to 130 J/mol for the 40 mol% CHOL membrane. When compared with RT (2,400 J/mol), the thermal energy per mole, we see that a relatively small amount of energy is required to cause irreversible failure by applied tensile stresses. The energy stored in the membrane up to failure is only 1.5–5.4% of the available thermal energy. This might be taken to imply that failure

results from a defect that comprises many (~50–100) molecules.

A simple thermodynamic interpretation along the lines of Whol (1946) can be made of the form of the toughness vs. membrane composition plot (Fig. 8) if we recognize that an increase in bilayer toughness represents an increase in stability and may be correlated with a decrease in overall free energy of the bilayer system. A decrease in the excess Gibbs energy of the bilayer due to mixing the two membrane components, namely, uncomplexed lipid and the lipid/cholesterol complex, would make a stabilizing contribution to the overall free energy. This simple analysis indicates a maximum in overall stability of the bilayer structure for bilayer cholesterol concentrations around 35 mol% CHOL. The experimental membrane toughness shows approximately the same form as the excess mixing free energy and gives some credence to the hypothesis that simply mixing the two postulated bilayer components confers stability on the structure. The enhanced stability, then, would appear to come from an increase in internal energy, i.e., an increase in the cohesive energy density of the material.

For the other systems, areas per molecule can be estimated from monolayer studies. BSM has a limiting area per molecule of ~42 A^2 (Shah and Schulman, 1967). This value is already close to the area per molecule of cholesterol and so, for the purpose of our toughness calculation, we may safely estimate an area per molecule for the BSM/CHOL 1:1 membrane of ~40 A^2. As given in Table 2, BSM/CHOL membranes have a toughness of 34–49 J/mol and, despite the higher modulus, are not as tough as SOPC/CHOL. Limiting areas per molecule for several highly unsaturated lipids have been measured by Demel et al. (1972b), from which we may estimate for DAPC a value of ~80 A^2. DAPC membranes were the least tough of all the membranes studied and the inclusion of cholesterol raised the toughness by only 50%.
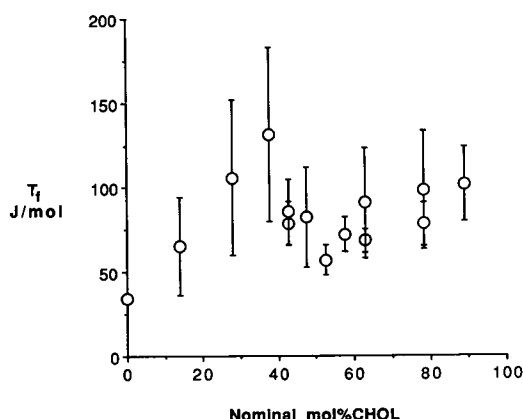
RBC lipid extract

The measurements made on extracted RBC lipids with native cholesterol can be used to give some insight into the origin of the natural, intact, RBC membrane compressibility if we again make an analogy with particulate composites, but this time identify lipid/cholesterol as the matrix component and integral membrane proteins as the less compressible particles.

Vesicle membranes reconstituted from extracted RBC lipids including native cholesterol (40 mol% CHOL) gave an elastic area expansion modulus of 423 dyn/cm. These reconstituted RBC lipid vesicles necessarily contained a symmetric distribution of lipids and, as is widely recognized, the native RBC membrane has an asymmetric distribution, although cholesterol appears to be symmetri-

FIGURE 8 Membrane toughness T_f vs. mol% CHOL for SOPC/CHOL system.

cally distributed (Lange et al., 1981). Previously measurements on different lipid types with similar acyl chain composition have shown that the presence of different head groups (phosphatidylcholine, -ethanolamine, and -serine, and even digalactosyl), do not significantly affect the area compressibility modulus (Evans and Needham, 1987). We therefore expect that, despite the loss of lipid asymmetry, the measured modulus should be close to that of the RBC lipids plus native cholesterol, and is representative of the natural membrane.

The lipid and cholesterol matrix and the integral transmembrane protein particles each occupy a certain fraction of the membrane area and, under conditions of isostress, the elastic modulus for the composite RBC membrane K_{RBC} is given by,

$$K_{RBC} = \left\{ \frac{a_{L\&C}}{K_{L\&C}} + \frac{a_P}{K_P} \right\}^{-1}, \tag{3}$$

where $K_{L\&C}$ and K_P are the elastic moduli for the lipid and cholesterol and protein components and $a_{L\&C}$ and a_P are their respective area fractions. As before, the simple property-averaging model assumes that the tensile stress is distributed evenly in the two components and that the total strain is a linear combination of the strains of each component multiplied by their respective area fractions. It also requires that the stress is continuous across the boundaries between the lipid matrix and the protein particles.

It is expected that the area compressibility of a membrane protein would be very high in comparison to the lipid cholesterol bilayer, and so a_P/K_P is essentially zero. If we also assume that the area fraction occupied by the lipid and proteins are each 0.5, then from our measurement of the elastic modulus of the extracted RBC lipid we would calculate from Eq. 3 the elastic area expansion modulus for a lipid/cholesterol membrane containing 50 area% integral membrane protein to be 940 dyn/cm, i.e., the highly incompressible membrane proteins themselves do not make a significant contribution to the membrane compressibility. When this result is compared with Katnik and Waugh's (1990) value for the intact RBC membrane (~500 dyn/cm), the apparent enhancement of compressibility of the lipid/cholesterol bilayer by the highly incompressible protein component would appear to come from an interaction with its surrounding annular lipid.

CONCLUDING REMARKS

The pattern that emerges from this and previous studies is that the presence of cholesterol is the single most influential factor in increasing bilayer cohesion, but only for lipids where both chains are saturated, mono- or diunsaturated, or where one chain contains multiple double bonds and the other is relatively unsaturated. Multiple unsaturation in both lipid chains inhibits the condensing effect of cholesterol in bilayers as well as monolayers. There appears to be a strong requirement for the hydrocarbon chains in the outer regions of the bilayer to go all-*trans*, at least in one of the chains, in order to pack with the ring structures of the planar rings of the cholesterol molecule (McIntosh, 1978; Yeagle et al., 1987).

By postulating the formation of a lipid/cholesterol complex and by considering the bilayer phase as a simple matrix-particulate composite composed of uncomplexed lipid and lipid/cholesterol complex, the experimentally determined elastic area modulus was modeled by an average of the two components scaled by their respective area fractions. The best fit to the data by this model implies a stoichiometry for the purported lipid/cholesterol complex of 1/1.22, i.e., each cholesterol molecule therefore, directly interacts with ~0.82 lipids. This stoichiometry is slightly different from the generally accepted limit of 1/1. Other studies that have investigated structural effects up to high nominal cholesterol concentrations also show a saturation limit for the property being measured. The magnetization exchange rate for egg PC/cholesterol membranes showed a similar nonlinear increase with increasing cholesterol and saturated at ~60 mol% CHOL (Fralix et al., 1990). Techniques that can precisely assay for the presence of cholesterol in the bilayer and the appearance of cholesterol crystallites in the aqueous phase (e.g., x-ray diffraction, NMR) on a series of lipids containing increasing numbers of double bonds per chain might reveal a range of limiting stoichiometries for the interaction of different lipids with cholesterol.

Despite the increase in both area modulus and tensile strength with increasing cholesterol concentration, it was found that bilayers containing intermediate amounts of cholesterol ~40 mol% CHOL, were the toughest. This was due to a proportionately larger increase followed by a decrease in the critical areal strain. The failure energy per mole was measured to be only a fraction of the available thermal energy per mole, implying that many molecules (50–100) may be involved in forming a critical-sized defect that ultimately results in failure. These data, along with a previous report of the synergy between tensile and electrocompressive stresses (Needham and Hochmuth, 1989), should help to guide further theoretical analyses that attempt to predict membrane instability phenomena arising from perturbation of the bilayer structure (Winterhalter and Helfrich, 1987).

The results presented in this paper demonstrate that for the simple lipid bilayer structure a wide range of compressibility, tensile strength, and toughness can occur depend-

ing on lipid type, cholesterol concentration, and the nature of the lipid/cholesterol interaction. Membranes with little cholesterol content and made from lipids with multiple double bonds are inherently weak, whereas the interaction of cholesterol with monounsatruated lipids can cause a dramatic increase in the area expansion modulus and strength of the bilayer membrane. The specific interaction of cholesterol with sphingomyelin produces a bilayer with the highest elastic modulus yet measured. Thus, in studies of natural and artificial membranes it is possible to precisely control the mechanical properties of the lipid bilayer by judiciously choosing the lipid and lipid/cholesterol composition. Elastic area expansion moduli can be made to cover a range from 50 to ~2,000 dyn/cm and tensile strengths can be made to vary from 2 to 30 dyn/cm. These lipid and lipid/cholesterol systems also extend the range of membrane stiffness which is useful in tests of current theories regarding membrane bending (Evans and Rawicz, 1990), and the results allow quantitative analyses to be made in studies concerning many forms of membrane breakdown and cell damage (Needham and Hochmuth, 1989).

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