CHOLESTEROL-INDUCED EFFECTS ON THE VISCOELASTICITY OF MONOGLYCERIDE BILAYERS

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ABSTRACT Changes in the viscoelastic properties of glycerol monooleate bilayers resulting from the incorporation of cholesterol into the membranes have been measured. The interface tension increases with the cholesterol concentration, reaching saturation for a 4.2:1 mole ratio of cholesterol:lipid in the film-forming solution. Incorporation of cholesterol in the membrane causes the appearance of a large intrinsic viscosity; this also increases with the sterol content of the membrane. Molecular models of lipid-sterol interactions and packing are considered to explain both the observed changes in membrane properties and similarities with comparable lipid systems.

INTRODUCTION

The morphology of bimolecular lipid membranes (BLM) and some of their properties attest to their close structural similarities to real biomembranes. Furthermore, synthetic membranes can be modified by the addition of various chemical agents so as to mimic other important aspects of biomembrane behavior. In this way the effects of chemical modifiers upon simple BLM can provide information on corresponding effects in biomembranes. Recently there has been considerable interest in the interaction of sterols with membrane lipids. Cholesterol is the most important biological sterol and in many biomembranes it is present in almost the same concentration as the lipids (e.g., 0.9 mole ratio of cholesterol to lipid in erythrocyte membranes).

The properties of lipid-cholesterol bilayers and multilamellar dispersions have been extensively studied. The physical techniques used have included ESR (1), NMR (2), x-ray diffraction and DSC (differential scanning calorimetry) (3), fluorescence polarization spectroscopy (4), and neutron diffraction (5). Modifications of the physical properties of both natural and synthetic lipid membranes induced by the incorporation of cholesterol have been known for some time. The reduction in fluidity, or equivalently the increase in order, within the membrane, which results from the inclusion of cholesterol, has been inferred from measurements of the fluidity of the hydrocarbon interior of the membrane (6) and from water permeability studies (7). Cholesterol at 1:6 mole ratio in lecithin membranes increases the order in the membrane interior both above and below the lipid phase transition (8). Shinitzky et al. (9) found an increase in the microviscosity from 1.61 to 3.57 cP for a twofold increase in cholesterol in CAB (cetyltrimethylammonium bromide) micellar systems. The change in membrane viscosity appears to parallel changes in the fluidity of the polymethylene chains of the lipid molecules. Studies using x-ray diffraction (3), ESR (1), and NMR (2) suggest that cholesterol actually inhibits the motion or degree of rotational freedom of the lipid chains, inducing ordering and hence reducing the overall fluidity of the entire membrane structure. The effects of cholesterol in lipid membranes can be attributed on the microscopic level to both the strong hydrophobic interaction of cholesterol with lipid and to the inherent rigidity of the cholesterol molecule.

Membrane viscosities have hitherto been deduced from measurements of diffusion constants of molecular probes in the membrane plane. The applicability of the concept of viscosity within membrane systems has recently been defined within the context of a mathematical treatment of two-dimensional rotational and translational diffusion (10). However, in most studies a complete analogy between the hydrocarbon membrane interior and a bulk fluid has been assumed to hold. Although clearly this must be an oversimplication, results of a wide range of experiments are usually interpreted in terms of a "microviscosity" having dimensions of a bulk viscosity. The term microviscosity reflects the effect upon the motion of molecular probes of local interactions within the membrane. The assumption stated above, whilst permitting the application of the usual diffusion equations (11), is clearly an idealization as BLM are more ordered than the isotropic fluids. Because viscosity is a bulk property of fluids, care must be exercized in the interpretation of viscosity values estimated by a particular technique.

Study of the changes in the macroscopic viscoelastic properties of the BLM, resulting from the incorporation of cholesterol, could provide additional information about the lipid-sterol molecular interactions. Often the macroscopic consequences of the presence of cholesterol in membranes are more significant than is the microscopic understanding of these effects (12).

The technique of laser-light scattering from thermally excited capillary waves on membranes (specifically using

photon correlation) is sensitive to composition-induced changes in the macroscopic viscoelastic properties of simple monoglyceride membranes (13). The present paper concerns the use of these methods to detect changes induced in similar BLM following the addition of cholesterol to the membrane-forming solutions.

The only viscosity of a symmetric membrane (defined as one adjoined by identical fluids) that is accessible to light-scattering experiments is that which influences capillary or transverse excitations (13). This membrane viscosity is associated with the dynamic component (γ') of the interfacial tension $\gamma(=\gamma_0 - i\omega\gamma')$. This property ($\gamma'$) is a true interfacial viscosity and can be identified as the transverse shear viscosity acting normal to the membrane plane (14). This membrane viscosity, not hitherto determined for BLM, is not strictly comparable with viscosities estimated by other techniques (4, 15).

The spectrum of the light scattered by thermally excited waves upon a symmetric membrane having nonzero γ' reflects the physical properties of the system (13). This spectrum is approximately Lorentzian. To first order the central frequency (ω_0) and the line width (Γ) of the spectrum can be related to the tension (γ_0) and the viscosity of the adjoining fluid (η) via

$$\omega_0 = (\gamma_0 q^3/2\rho)^{1/2} \quad (1)$$

and

$$\Gamma = \eta q^2/\rho. \quad (2)$$

MATERIALS AND METHODS

Membranes

Bilayer membranes were formed from solutions comprising 5 mg glycerol monooleate (GMO) per ml of *n*-decane. The details of the methods have been described elsewhere (13). The fluid bathing the membranes consisted of aqueous 0.1 M NaCl, buffered to pH 6.0. Chromatographically pure cholesterol (>99%) was obtained from Sigma Chemical Co. (St. Louis, MO): no further purification was attempted. The cholesterol was weighted out with a precision microbalance (Mettler Instrumente, Zurich, Switzerland), appropriate volumes of solution being used to give final concentrations of 5, 10, 15, and 21 mg cholesterol per ml of solution. Cholesterol does not readily dissolve in *n*-decane. The dissolution process was promoted by warming the vessel containing the solution of lipid plus cholesterol with the hands and agitating it until all the cholesterol crystallites had completely disappeared. At concentrations >21 mg/ml, white crystals of cholesterol were visible in the Plateau-Gibbs border of the membranes. Such membranes were unstable and ruptured shortly after thinning to the black state. This concentration apparently corresponded to saturation with cholesterol of the final bilayer membrane.

The formation characteristics of cholesterol-containing GMO/*n*-decane membrane differ in certain respects from those of membranes containing only lipid. Initially, the newly formed film was thick and slow draining. However, upon the appearance of an optically black region at the margin, the transition to the bimolecular membrane ensued extremely rapidly and within a few seconds the entire membrane was black and transparent. This behavior resembles the thinning of solvent-free membranes formed from dispersions of GMO in squalane (13). This observation suggests that there may be some similarities between the viscoelastic properties of the two membrane types.

The cholesteric membranes appeared visibly rigid, judged by their comparatively weak response to mechanical disturbances. The relative insensitivity of these membranes to mechanical oscillations reduced the possibility of serious errors in the estimation, by light scattering, of the damping coefficient of the capillary waves (13).

Light Scattering and Data Interpretation

The light-scattering experimental arrangement has been described previously (13). Photon correlation functions were recorded with a 96-channel single-clipping Malvern correlator; typical data accumulation times were between 2 and 5 min, depending on the wave vector of the capillary mode studied. This wave vector (**q**) was defined by the angle of scattering:

$$\mathbf{q} = \mathbf{k}_0 - \mathbf{k}_s$$

where $\mathbf{k}_0$ and $\mathbf{k}_s$ are the wave vectors of the specularly reflected and the scattered light, respectively.

The thermally excited capillary waves of lipid-cholesterol BLM led to correlation functions that invariably comprized damped oscillations (Fig. 1). Despite the inherent rigidity of such BLM, no transition to overdamped motion was ever observed, indicating that conditions of low damping were always applicable. The observed correlation functions were fitted to an appropriate analytic form:

$$G(t) = A + B\cos(\omega_0 t + \phi)\exp(-\Gamma t - \beta^2 t^2/4) \quad (3)$$

to derive values of ω_0 (the frequency of the capillary wave) and Γ (its damping coefficient). For a given q value these parameters are related to the physical properties of the BLM through the appropriate dispersion equation. Instrumental effects were allowed for in the data fitting by the parameter β. The phase term ϕ accounted for the deviations of the observed spectrum of the scattered light from a Lorentzian form. Omission of either or both of these parameters from the fitting procedure can lead to serious errors (13).

RESULTS

Frequencies and damping coefficients measured for membranes of all cholesterol concentrations are shown in Fig. 2.

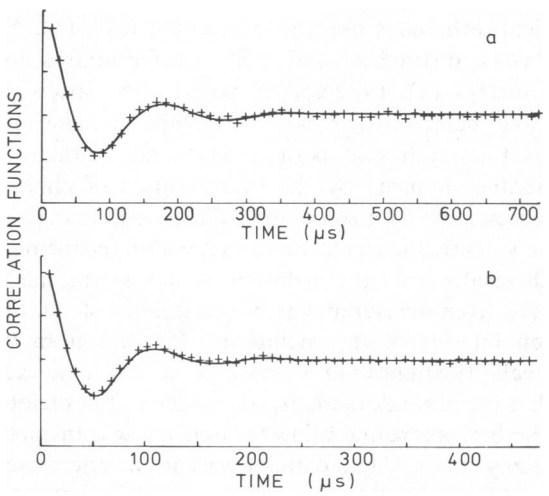

FIGURE 1 Typical autocorrelation functions observed at q of (*a*) 790 cm^{-1} and (*b*) 1,129 cm^{-1}, for a BLM formed from a solution saturated with cholesterol. Note the different time scales. The curves represent the best fit functions using Eq. 3. Such BLM are characterized by low noise correlation functions.

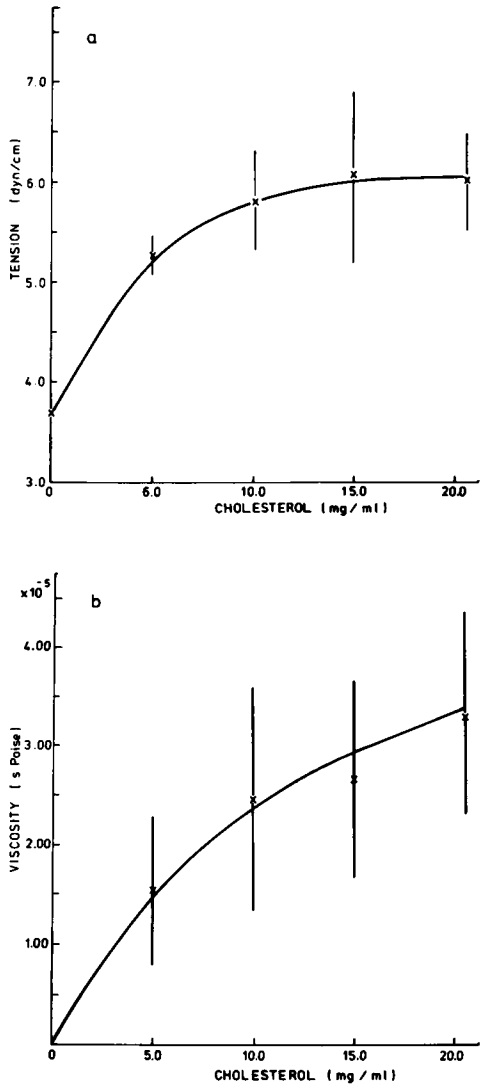

FIGURE 2 Capillary wave frequency (*a*) and damping constant (*b*) vs. q. Data for film-forming solutions containing 5(O), 10(+), 15(×), and 21(●) mg/ml cholesterol are shown, together with theoretical dispersion curves based on the appropriate mean membrane tension and viscosity (---). The solid lines (—), corresponding to BLM containing no cholesterol, are shown for comparison. The increase in ω_0 and Γ with cholesterol content indicates the increase in membrane tension (Eq. 1) and apparent viscosity of the bathing solution (Eq. 2).

Both ω_0 and Γ increase as the cholesterol concentration increases.

Approximate expressions for the roots of the dispersion equation for capillary waves have been derived elsewhere. These expressions connect ω_0 and Γ, the measured parameters, with the physical properties of the system: γ_0 and γ' of the membrane and η of the aqueous phase. The simple approximations of Eqs. 1 and 2 have been extended to include, for example, the effect of γ' (13).

If the membrane viscosity γ' is assumed to be zero, the observed values of ω_0 and Γ yield initial estimates of the interface tension and the bathing solution viscosity using the analytic approximations for the complex frequency $\omega(= \omega_0 - i\Gamma)$. At all wave vectors studied for cholesterol-containing membranes, the apparent viscosity of the aqueous medium exceeded the accepted value (1.025 cP) for 0.1 M NaCl (13). The magnitude of the apparent viscosity increased systematically with the cholesterol concentration in the parent membrane-forming solution, reaching 1.69 ± 0.13 cP for the 21 mg/ml case. Such excessive viscosity values contrast with the value of 1.07 ± 0.15 cP observed for 5 mg/ml GMO/*n*-decane membranes containing no cholesterol (13).

The excessive damping of the observed correlation functions was interpreted in terms of the transverse shear viscosity γ'. Although analytic approximations for ω_0 and Γ incorporating the effect of γ' have been derived (13), they are of only limited validity because of the strong coupling between γ_0 and γ', particularly at large values of the latter. More exact estimates of γ_0 and γ' can be found by direct solution of the dispersion equation, substituting the fitted values of ω_0 and Γ for the real and imaginary parts of ω. The appropriate dispersion equation can be parameterized as (13)

$$(S^2 + Y + \tau'S)(1 + 2S)^{1/2} - (Y + \tau'S) = 0 \qquad (4)$$

using the reduced quantities

$$S = -i\omega\tau, \tau = \frac{\rho}{2\eta q^2}, Y = \frac{\gamma_0 \rho}{8\eta^2 q}, \tau' = \frac{\gamma' q}{4\eta}. \qquad (5)$$

The real and imaginary parts of Eq. 4 form simultaneous homogeneous equations, which were solved numerically to yield values of γ_0 and γ'. In these solutions η was assumed to be 1.025 cP.

Values of γ_0 and γ' found thus were averaged over all wave vectors studied and over several membranes formed from similar solutions to avoid the effects of fluctuations in BLM composition and inherent experimental variability (13).

The average interface tension increased from 3.64 ± 0.22 dyn/cm for an unmodified GMO membrane to 6.06 ± 0.49 dyn/cm for 21 mg/ml cholesterol concentration. The transverse shear viscosity of the membrane, which is undiscernible for the pure GMO/*n*-decane membrane, rises from 1.57 ± 0.80 × 10^{-5} sP (surface poise or dyn s/cm) to 3.35 ± 1.27 × 10^{-5} sP as the cholesterol content rises from 5 mg/ml to saturation (21 mg/ml). The large errors in γ' reflect the lower precision of determination of Γ compared with ω_0 in photon correlation experiments.

The dispersion curves shown in Fig. 2 are based upon the accepted value of η and the average values of γ_0 and γ' for each membrane type. The dispersion curves for unmodified membranes formed from solutions of 5 mg/ml GMO in *n*-decane are shown for comparison. The convergence of the dispersion curves at increasing cholesterol concentrations is an obvious feature of Fig. 2, suggesting that the effect of cholesterol upon the viscoelastic properties of the

BLM may saturate. This tendency is emphasized in Fig. 3; saturation is apparently observed at cholesterol concentrations of 21 mg/ml. Both γ_0 and γ' appear to behave similarly. The highest value of γ' observed, 3.35 ($\pm$ 1.27) $\times$ 10^{-5} sP for 21 mg/ml cholesterol content, is very similar to values measured for solvent-free membranes of GMO (13), 3.8 ($\pm$ 1.4) $\times$ 10^{-5} sP, and for fully condensed monolayers of GMO supported upon an aqueous subphase (16), 3.5 ($\pm$ 1.4) $\times$ 10^{-5} sP. Although the membrane viscosity reached comparatively high values, no transition to overdamped fluid motion was observed for these membranes because of the concomitant increase in the tension.

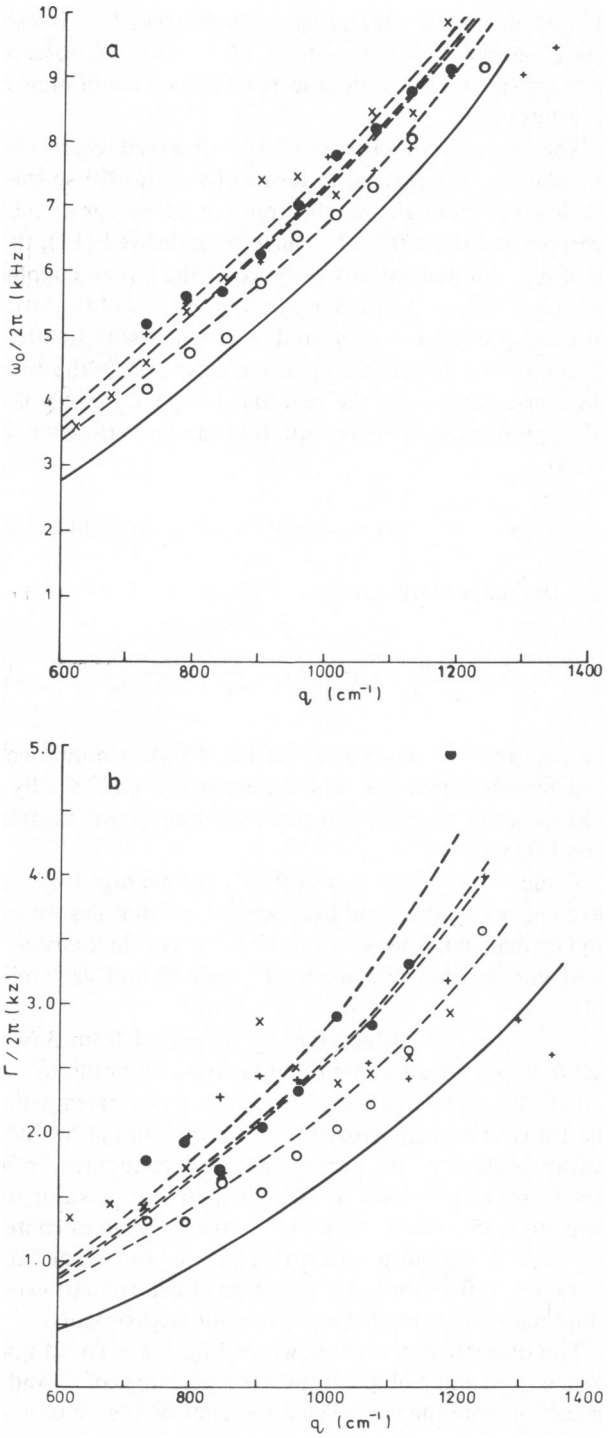

FIGURE 3 Data for membrane tension (*a*) and tranverse shear viscosity (*b*) at various cholesterol concentrations. The curves are purely to guide the eye. The error bars correspond to standard deviations. The greater uncertainties for individual γ' values make the exact behavior of γ' with cholesterol concentration unclear.

DISCUSSION

The results presented here represent the first quantitative estimates of the viscoelastic parameters of monoglyceride BLM modified by the addition of cholesterol. These studies provide a macroscopic approach to the further understanding of the lipid-sterol interaction. Although these results permit some definitive assertions concerning changes in macroscopic membrane properties to be made, the exact interpretation of the variation of membrane viscoelasticity with cholesterol concentration in terms of molecular interaction within the BLM is not at all obvious.

Before presenting possible interpretations of these results, the main experimental uncertainties must be considered. The actual concentration of cholesterol within the BLM can be varied only indirectly through the composition of the bulk membrane-forming solution. The exact composition relationship between the bilayer and the membrane-forming solution has not been verified experimentally in this work. In the present work the mole ratio of cholesterol to lipid in the parent solution that appears to correspond to saturation in the ultimate lipid bilayer (4.25:1) agrees well with the composition studies of Pagano et al. (17). However, although saturation can be achieved without difficulty, the cholesterol content of the BLM cannot be controlled exactly. This fact may explain the large spread in the estimated values of tension and viscosity, particularly at the higher cholesterol concentrations. The uncertainties associated with the light-scattering method are likely to be less significant (13).

Although various instrumental factors could increase the estimated damping constants of the capillary waves it is highly improbable that the observed increase in both tension and intrinsic viscosity of the membrane could result from such effects. The systematic increase of both these properties with cholesterol concentration would be uncharacteristic of an instrumental effect. As pointed out by Grabowski and Cowen (18), instrumental line broadening may cause a decrease in the estimated tension with an increase in the apparent viscosity. Also, for the range of wave vectors studied, a 65% increase in the damping coefficients would require an instrumental line width on the order of 1,000 cm^{-1}. Such an instrumental function seems most unlikely in view of previous results (13). Finally, the variation of the estimated properties with cholesterol concentration shows the saturation effect that is typical of other properties of lipid-cholesterol BLM (7, 19). The effects of cholesterol on the viscoelastic prop-

erties of simple monoglyceride BLM seems indeed to be real.

Cholesterol-containing BLM formed from a GMO-*n*-decane solution are three component systems in which interfacial adsorption is likely to be complicated. It is, therefore, difficult to find a simple explanation for the observed data on both interfacial tension and viscosity. The main difficulty is to reconcile the simultaneous increase in both these properties with cholesterol concentration. An increase in tension would indicate that cholesterol apparently reduces the interfacial adsorption in contrast with the findings of Pagano et al. (17) of a decrease in the average area per lipid molecule signifying a condensing effect of the sterol in GMO bilayers. However, a condensation results in a stronger cohesive force within the bilayer, which would lead to an increased interfacial viscosity. An increased viscosity is compatible with observations of other physical measurements (1, 2). Thus, taken separately, the increase in tension and the increase in viscosity with cholesterol concentration can both be explained. However, a complete and consistent explanation of both experimental results is not possible in terms of any simple molecular model.

In the BLM the cholesterol molecule is oriented with its hydroxyl group at the aqueous interfaces where it occupies a considerable interfacial area, thus spacing out the polar groups of the host GMO molecules. The cholesterol molecules have bulky, nonplanar cross sections, and probably constitute obstacles to the compact packing of the acyl chains of the GMO molecules. Therefore, the increase in the tension with cholesterol concentration may be the result of a reduction in the concentration of adsorbed GMO molecules at the BLM interfaces. The rigid steroid nucleus of the cholesterol molecule is interdigitated between the alkene chains of the GMO molecules (20). The increase in the membrane interfacial viscosity can be attributed to a reduction in the fluidity of the membrane interior since cholesterol inhibits the normal rotational motion of the alkyl chains (1, 2). As the cholesterol concentration in the bilayer increases, the space available at the interfaces for the GMO molecules is reduced, and at a concentration corresponding to saturation the interfacial adsorption of the lipid is minimal. A decrease in the concentration of GMO molecules at the BLM interfaces would indeed give rise to the observed increase in tension. However, if the only effect of cholesterol were to disrupt the lipid molecular arrangement this model would not explain the observed increase in membrane viscosity. The strong hydrophobic interaction of the cholesterol ring system with the proximal part of the GMO alkene chain inhibits the motion of that part of the GMO molecule. Thus close to the interfaces, where the hydrophilic groups reside, the BLM becomes rather less fluid, whereas in the membrane interior, containing the distal part of the GMO alkene chain along with the short alkane chain of cholesterol, the structure remains comparatively more fluid.

These ideas may explain why the transverse shear viscosity (which is an interfacial viscosity) is affected so dramatically by the presence of cholesterol. The differential inhibitory effect that this model assumes results from the tapered steric profile of the cholesterol molecule itself. This concept has been invoked (21) to explain certain anomalies in experimental results obtained by different techniques. Thus, on the basis of this model, the increase in interfacial shear viscosity (γ') can be attributed to the conformational inflexibility of the rigid ring structure of cholesterol that partially immobilizes those parts of the GMO chains close to the glycerol backbone. However, even a BLM saturated with cholesterol retains some fluidity in its interior since the short hydrocarbon chain of cholesterol that parallels the terminal portion of the GMO molecules enhances the degree of rotational freedom of this part of the lipid chain (21).

The remarkable similarity of the value of viscosity for cholesterol-saturated GMO membranes to that for both solvent-free bilayers (13) and fully condensed GMO monolayers on an aqueous subphase (16) suggests that this viscosity value is associated with the interaction between GMO molecules. It is not unreasonable that these viscosities could arise from a solvent exclusion effect. Other experimental studies suggest a mechanism by which cholesterol could lead to solvent exclusion in GMO bilayers. Due to the location of the double bond in the GMO molecule at carbon atom 9, exactly in the center of the chain, the GMO acyl chain favors the *cis*-bond, or bent configuration (22). Normal decane has a chain length equal to the distal portion of the GMO chain. The interactive volume generated within bilayers of GMO by the motion of the alkene chain is maximal for *n*-decane. Thus, in bilayers formed from GMO in *n*-decane the molecules adopt a more extended configuration giving these BLM maximum possible thickness. The maximal interactive volume for GMO molecules in membranes incorporating *n*-decane (but no cholesterol) allows maximum rotational freedom for the lipid chains. Such BLM exhibit no discernible membrane viscosity (13). The introduction of cholesterol into the bilayer clearly reduces the magnitude of the interactive volume so that the GMO acyl chains cannot extend as fully as in the absence of cholesterol. Because the cholesterol molecule, which is equivalent in length to a hydrocarbon chain of fourteen carbon atoms (compared with 18 for GMO), straddles the double bond position of the GMO alkene chain, the interactive volume available to be occupied by *n*-decane is reduced. Thus, as pointed out in a previous publication (13), the lipid chain interaction is enhanced due to the comparative absence of solvent.

This hypothesis is supported, at least qualitatively, by the thinning characteristics of films formed from cholesterol-containing GMO *n*-decane solutions. The transition to the ultimate black state is very rapid and the solvent is seen to be physically squeezed out of the film by the advancing black region of the film. The thinning behavior of lipid-cholesterol membranes was remarkably similar to that

observed for solvent-free BLM of glycerol monooleate (13). The final state BLM appeared considerably less reflective than in the absence of cholesterol; no large visible lenses were ever observed. These observations strongly suggest that the inclusion of cholesterol in GMO-*n*-decane membranes does lead to an overall reduction in the solvent content of the BLM. From their experimental results on molecular areas in mixed cholesterol-GMO bilayers, Pagano et al. (17) concluded that the cholesterol ring system must occupy some of the internal volume normally available to solvent molecules and oleyl side chains of GMO. It follows, therefore, that with increasing cholesterol concentration in the bulk membrane-forming solution the concentration of cholesterol in the ultimate bilayer increases at the expense of both the lipid and solvent content of the BLM.

The data presented here indicate the extent to which cholesterol modifies the viscoelasticity of chemically simple bilayer membranes. From a detailed discussion of possible molecular interpretations of these results, it is clear that only careful consideration of the unique geometry and steric profile of the cholesterol molecule itself can lead to a consistent explanation of all the data.

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