INTRODUCTION

Myelin is a lipid-protein membrane characteristic of nervous tissue that spirals in a multi-layered sheath structure around axons. Its function is to promote fast and efficient conduction of nerve impulses (Morell, 1984). Myelin distinguishes itself from other membranes by its large proportion of lipids, 70% by weight (Norton and Cammer, 1984). Cholesterol (Chol) and sphingomyelin (SM) account for a large portion of these lipids (Table 1) and are mainly found at the extracellular side of the myelin membrane; 66% of Chol and practically all of SM are found extracellularly (Morell, 1984).

Table 1. Lipid composition of normal human brain myelin, as detected with evaporative light scattering high pressure liquid chromatography (Ohler et al., 2001).

Lipid	Mass %
Cholesterol (CHOL)	31.9
Phosphatidylethanolamine (PE)	19.2
Phosphatidylcholine (PC)	14.4
Hydroxy-Galactocerebroside (HCer)	10.8
Sphingomyelin (SM)	10.2
Cerebroside Sulfates (SCer)	6.7
Non-Hydroxy Galactocerebroside (NCer)	4.0
Phosphatidylserine (PS)	1.5
Phosphatidylinositol (PI)	trace

Multiple Sclerosis (MS) is the most common demyelinating disorder of young adults (Compston and Coles, 2002). In MS, demyelination appears to occur by the loss of the compact multilamellar structure and the formation of large vacuoles that appear to degrade into smaller vesicles (Genain et al., 1999; Raine et al., 1999). The splitting at the

extracellular side of the membrane implies a loss of intermembrane adhesion (Genain et al., 1999).

While many researchers emphasize the role of proteins in intermembrane adhesion (Williams and Deber, 1993; Boison et al., 1995), it has not been proven that protein changes cause demyelination in MS (Seboun et al., 1999), with the possible exception of an alteration in myelin basic protein (MBP) in the (rare) Marburg variant of MS (Beniac et al., 1999). In our laboratory we have investigated the role of lipids in myelin structure. Their unusual abundance and clearly asymmetric distribution across the myelin bilayers serve as arguments for their important roles. Indeed it was found that in Experimental Allergic Encephalomyelitis (EAE) in the common marmoset, a relevant animal model of MS, the lipid composition of the myelin membrane was severely altered (Ohler et al., 2003, submitted). In particular, the concentration of SM was decreased 67%, whereas the concentration of Chol was increased 17%, changing the mass ratio of Chol:SM from 4:1 to 11:1 in EAE compared to controls (Ohler et al., 2003, submitted).

Numerous studies have indicated the existence of micro-domains enriched in SM and Chol in cell membranes (Brown and London, 2000**;** Dobrowsky, 2000). These domains, often referred to as rafts (Simons and Ikonen, 1997), are said to be important for a variety of cellular functions such as protein and lipid sorting (Verkade and Simons, 1997; Vinson et al., 2003). A disruption of these domains could therefore have major influence on the functioning of a membrane.

To build on the observation of an altered Chol:SM ratio in EAE (Ohler et al., 2003, submitted), we have explored in the current study the thermodynamic phase transitions, as measured by Langmuir isotherms, of a series Chol:SM mixtures centered around what is believed to be a critical lattice formation (Virtanen et al., 1995; Somerharju et al., 1999). The goal of this research was to investigate if changes in membrane lipid composition could affect the regular distribution of Chol:SM mixtures on a molecular level. A distortion in the regular distribution of lipids could potentially influence micro-domain formation (Somerharju et al., 1999) and the balance of fragile intermembrane forces.

Considerable literature exists on membrane lipids showing thermodynamic deviations at specific mole fractions of cholesterol, such as 0.22, 0.33 and 0.50 (Jain, 1988; Hui, 1989; Virtanen, 1995). Different explanations have been proposed for this behavior. One recent intriguing explanation is the formation of superlattices (Somerharju et al., 1985; Chong, 1994; Virtanen et al., 1995). At specific critical concentrations, cholesterol tends to distribute regularly in sterol-phospholipid membranes (Somerharju et al., 1999).

The superlattice model states the following (Virtanen et al., 1995): (i) phospholipid acyl chains exist in a lattice with hexagonal symmetry; (ii) each Chol molecule replaces a single chain in such a lattice; (iii) Chol molecules cause local lattice perturbations because of their larger cross-sectional area (Figure 1); (iv) the overall lattice perturbation energy is minimized when the Chol molecules are maximally separated from each other; (v) such maximal (and equal) separation is realized when the Chol molecules form a superlattice with hexagonal or centered-rectangular symmetry (Virtanen et al., 1995).

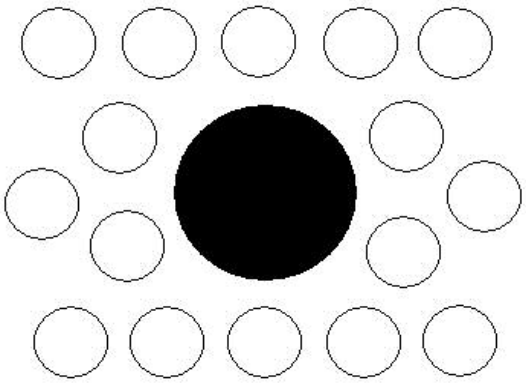

Fig. 1. Schematic presentation of a lateral lattice strain caused by a bulkier guest molecule. Insertion of a bulkier element, like cholesterol (black), into an acyl chain (white) lattice introduces strain that distorts the lattice rows. This strain decays in a manner that depends on temperature and the nature of the acvl chains etc.

From the superlattice model it follows that there is only a limited number of allowed critical concentrations for each component due to packing constraints. The critical compositions can be obtained from simple formulas such as:

$$Xcr = 2 / b^2 + 2ab + 2 - f \qquad (1)$$

Where Xcr is the critical guest lipid mole fraction (cholesterol) in a centered-rectangular superlattice, a and b are the coordinates of the guest molecules closest to the one at the origin. The parameter f is equal to the number of alkyl chains replaced by the guest in the lattice (Virtanen et al., 1995). Since each superlattice is thought to represent a local minimum of the membrane free energy, the membrane lipid composition would tend to

spontaneously settle on a value corresponding to a superlattice. Indeed it was observed that the phospholipid compositions of mammalian red blood cell membranes generally fall close to critical compositions predicted by the superlattice model (Virtanen et al., 1998). The superlattice model would be of particular interest for the myelin membrane because of the high lipid/protein ratio in this membrane and the fact that in demyelinating diseases, such as MS and EAE, the lipid composition is altered.

Based on previous literature results (Jain, 1988; Somerharju, 1999) we chose to study Chol:SM mixtures around three different Chol concentrations: 22.2 mol%, 33.3 mol% and 50.0 mol%. These specific concentrations are most often associated with abrupt physical changes in membrane properties (Jain, 1988; Hui, 1989; Virtanen et al., 1995). Furthermore, because previous studies showed that changes in acyl chain composition of SMs markedly influenced their in-plane elasticity (Li et al., 2000), we compared the packing of both completely saturated (egg derived) and partly unsaturated (bovine brain derived) SM with Chol over a range of compositions to explore the possibility that changes in Chol:SM could disrupt myelin domain formation in acute demyelination.

MATERIALS AND METHODS

Lipids Cholesterol, egg and bovine brain SM were purchased from Avanti Polar Lipids (Alabaster, AL). The acyl composition of egg SM consisted of 86% palmitate (16:0), 6.3% stearate (18:0), 1.8% arachidate (20:0), 3.8% behenate (22:0) and 4.2% lignocerate (24:0). The acyl composition of bovine brain SM consisted of 1.7% palmitate (16:0), 45.5%

stearate (18:0), 5.1% arachidate (20:0), 7.2% behenate (22:0), 6.0% lignocerate (24:0), 6.3% nervonate (24:1) and 28.2% other (un)saturated acyl chains. The lipids were dissolved in chloroform. Solutions of known concentrations were prepared gravimetrically, using a microbalance with a resolution of 1 μg. The lipids were weighed on small boats of aluminum foil which were thoroughly washed with solvent before use. The use of foil boats permitted the lipids to be thoroughly rinsed into the micro-scale volumetric flasks that were used. These stock solutions were typically 1-3mg/ml, from which they were subsequently mixed and diluted to about 0.3-0.4mg/ml for use on the trough such that 15-25 μl of solution of a micro syringe contained enough lipid to cover the trough at an appropriate area per molecule. When not in use the solutions were stored at -20˚C for no longer than four weeks. Mixtures of predicted superlattice concentrations, containing 22.2, 33.3 and 50.0 mol% Chol, were prepared, as well as mixtures with concentrations of 0.3 and 0.6 mol% more and 0.3 and 0.6 mol% less Chol than the predicted superlattice concentrations.

Langmuir monolayers The solution was deposited dropwise from a 25-μl syringe (Hamilton C. Reno, NV) to the ultrapure water subphase (Millipore PF Plus, resistivity 18.2 MΩ/cm, Millipore Corp., Bedford, MA) of a Nima 611 miniature Langmuir trough (Coventry, England) with a maximum area of 80 cm^2. After allowing 20 minutes for solvent evaporation, compression was commenced at 3 cm^2/min. This long equilibration time was found to improve the reproducibility of the isotherms. Surface pressure was measured with a Wilhelmy plate filter paper sensor (Nima Technology, Coventry, England). Pressure-area (Π-A) isotherms were measured at 22˚C (room temperature).

Analysis of Isotherms Mixing behavior of Chol:SM lipid monolayers was analyzed by mean molecular area-composition diagrams. Experimentally observed areas in the mixtures were compared with areas calculated by summing the molecular areas of the pure components (apportioned by mole fraction in the mix). The calculated mean molecular areas of the mixtures (A_π) were determined at a given surface pressure (π) using the following equation for ideal mixing:

$$A_\pi = X_1(A_1)_\pi + (1 - X_1)(A_2)_\pi \qquad (2)$$

Where X_1 is the mole fraction of component 1 and $(A_1)_\pi$ and $(A_2)_\pi$ are the mean molecular areas of pure components 1 and 2 at identical surface pressures. Negative deviations from mixing indicated condensation and implied intermolecular accommodation and/or dehydration interactions between lipids in the mixed films (Smaby et al., 1996).

Statistics For statistical analysis of isotherm data (Figure 3), three measurements were selected to represent each isotherm: the molecular areas A at Π = 10, 20 and 30 mN/m. These values are shown in Table 2. A two-tailed, unpaired *t-test* was used to test for statistical significance in the comparison of bovine brain SM and egg SM.

RESULTS

Figure 2 shows isotherms of cholesterol and bovine brain SM mixtures. It is clear that with increasing Chol content, the mean area per molecule decreases.

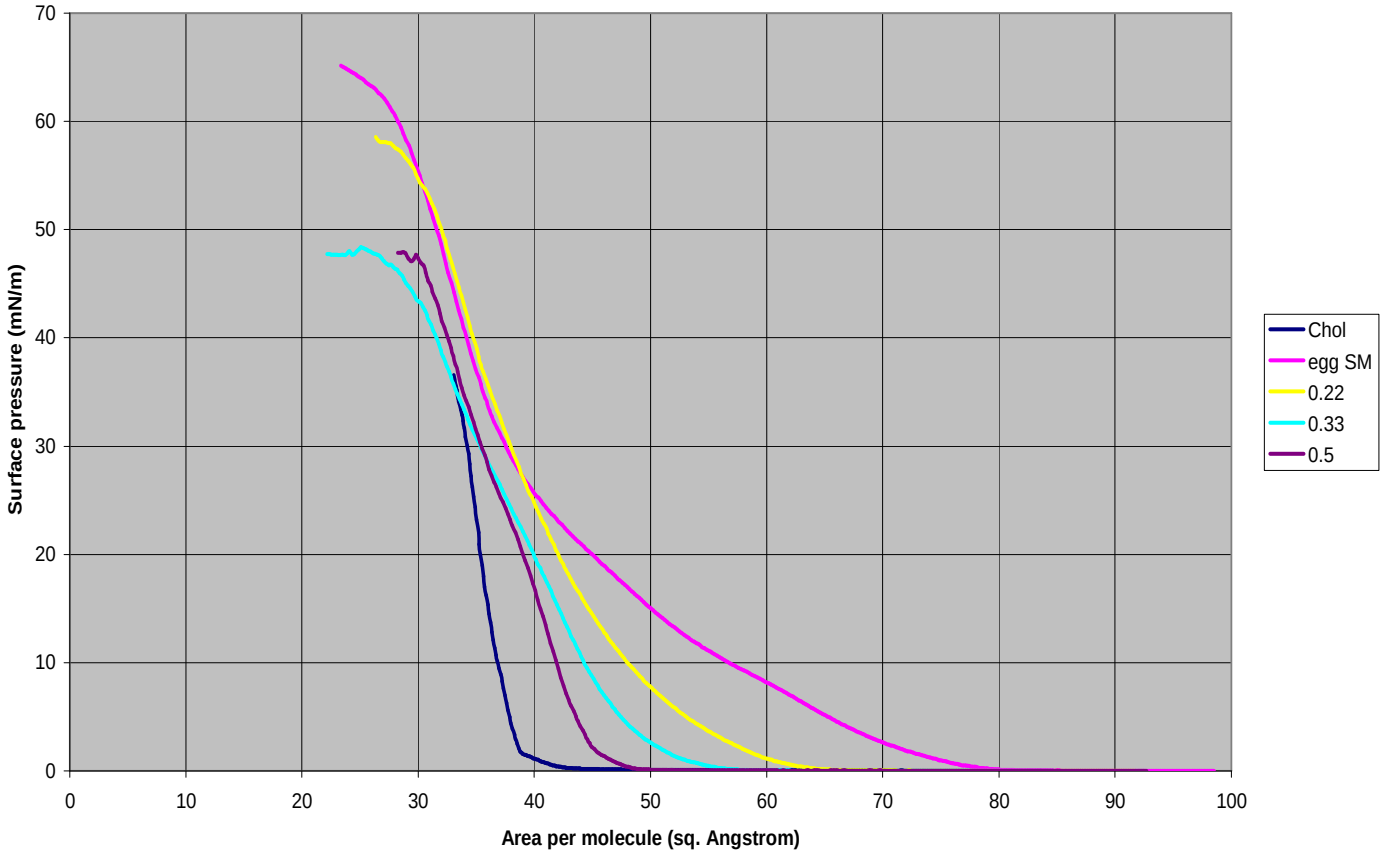

Figure 2: Pressure-area (Π-A) isotherms of Chol, egg SM and mixtures of egg SM-cholesterol on pure water at 22˚C. Cholesterol mole fractions are as indicated in the legend.

Figure 3 shows isotherms of bovine brain SM and egg SM. It is readily apparent that brain SM has a smaller area per molecule, although it contains more unsaturated *sn*-2 acyl chains compared to egg SM. The unsaturated to saturated ratio for bovine brain SM is 1:10, whereas egg SM is completely saturated.

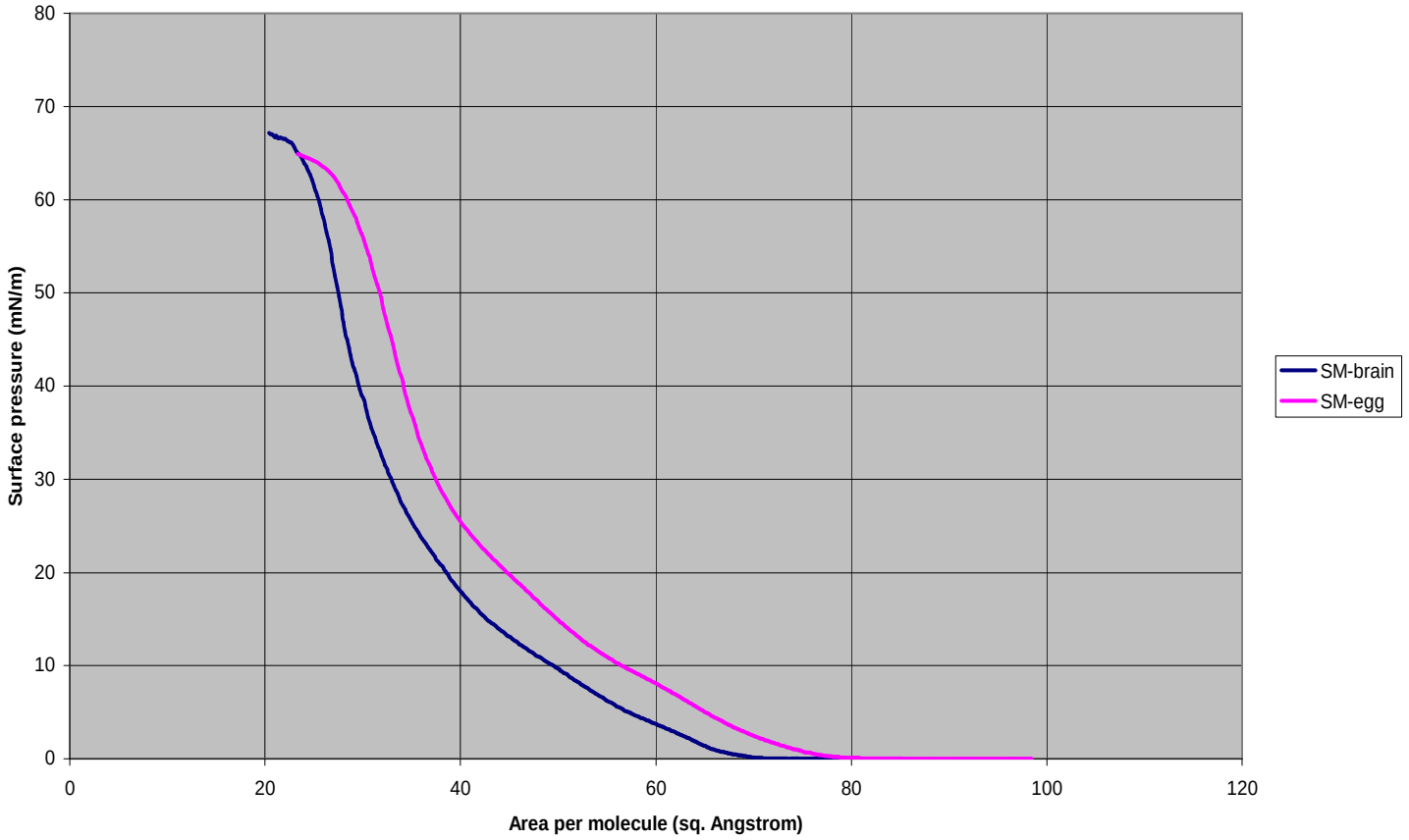

Figure 3: Pressure-area (Π-A) isotherms of bovine brain SM and egg SM on pure water at 22˚C

Three measures of the isotherms (the molecular areas A at Π = 10, 20 and 30 mN/m) indicated that this expansion was statistically significant (Table 2).

Table 2. Bovine brain SM and egg SM molecular areas, A, taken from isotherms in Figure 4 at various surface pressures, Π. Note that bovine brain SM areas at all pressures are decreased compared to the corresponding egg SM areas.

Pressure Π (mN/m)	Brain SM molecular area, A (Å^2) *	Egg SM molecular area, A (Å^2) *	Significance (*p*) #
10	56.4 ± 0.5	60.1 ± 0.3	<0.0006
20	42.4 ± 0.3	45.8 ± 0.3	<0.00005
30	35.5 ± 0.5	38.1 ± 0.5	<0.006

* Areas are expressed as an average of five measurements ± the standard error of the measurement.
# Two-tailed unpaired t-test confirmed significant differences between the isotherms of bovine brain SM and egg SM.

Figures 4A and 4B show the condensing effect of Chol on bovine brain and egg SM respectively at three different surface pressures (10, 20 and 30 mN/m). Solid lines represent ideal mixing of mean molecular areas at the designated surface pressures and were calculated using Equation 2 in the Materials and Methods.

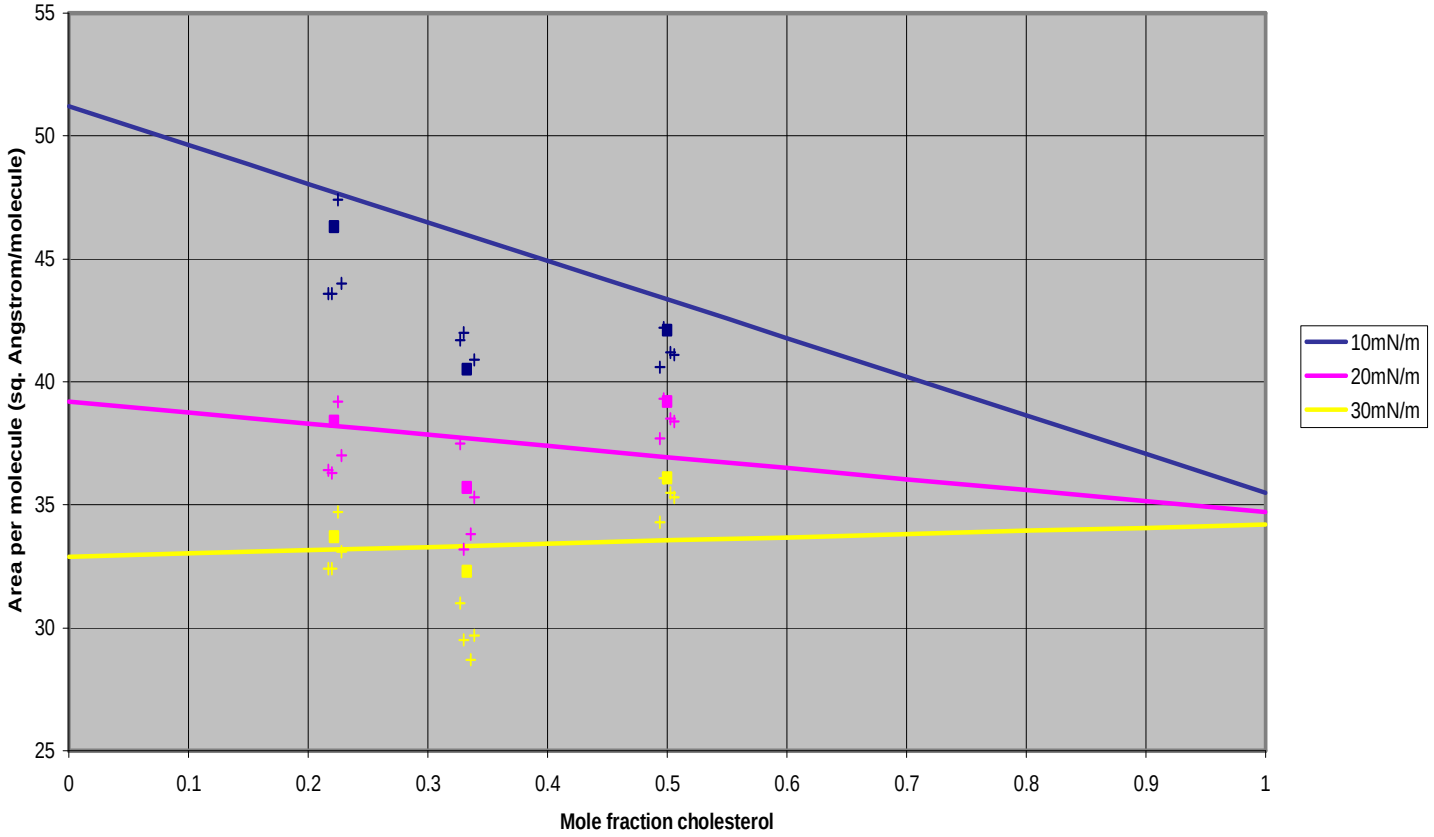

Figure 4A: Mean molecular area vs. composition. Experimental points are from the isotherms of Chol:brain SM mixtures. Crosses represent Chol mole fractions just around predicted critical concentrations of 0.22, 0.33 and 0.50 mole fractions. Squares are the exact Chol superlattice mole fractions of 0.22, 0.33 and 0.50. Solid lines represent ideal mixing of mean molecular areas at the designated surface pressures and were calculated using Equation 2 in Materials and Methods.

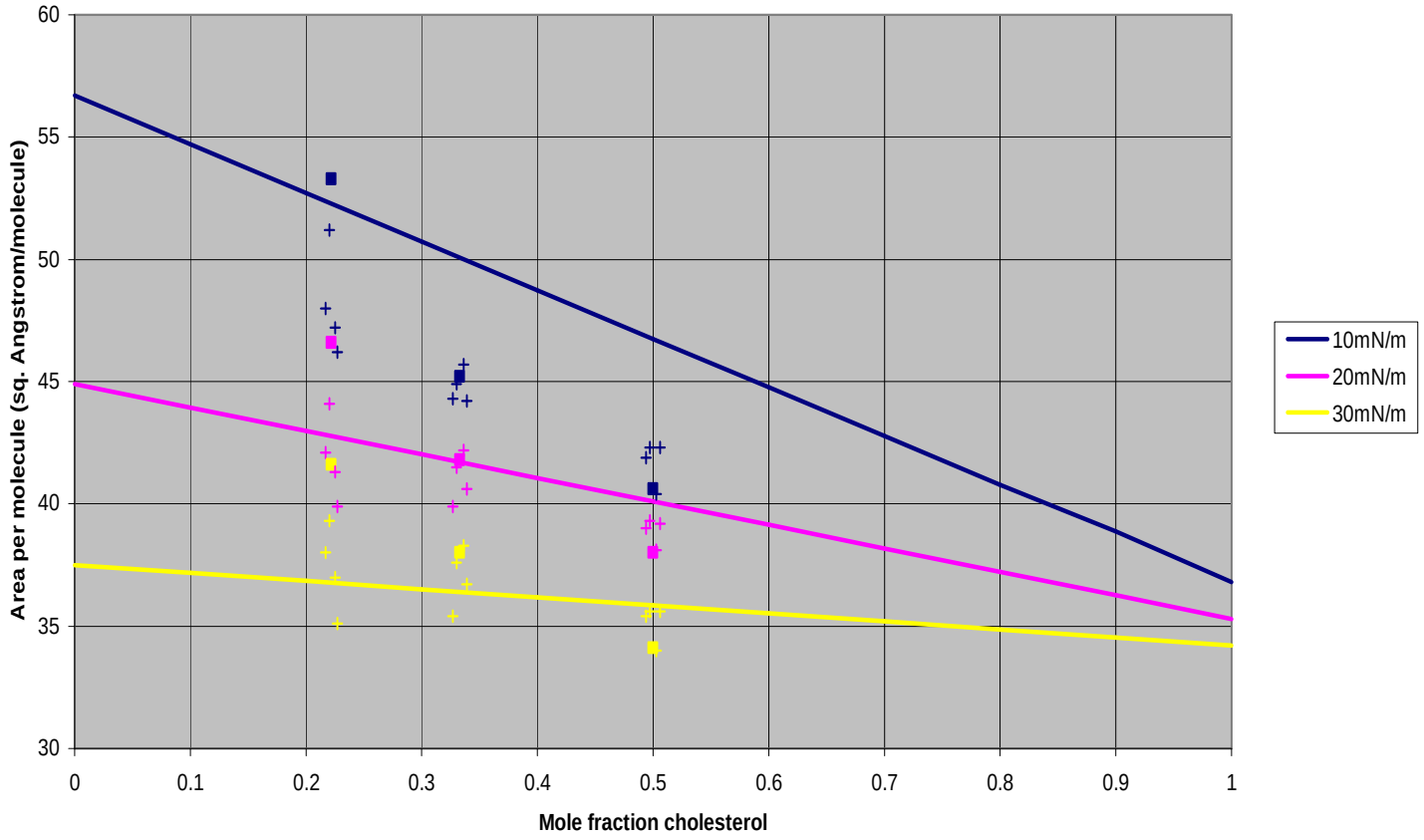

Figure 4B: Mean molecular area vs. composition. Experimental points are from the isotherms of Chol:egg SM mixtures. Crosses represent Chol mole fractions just around predicted critical concentrations of 0.22, 0.33 and 0.50 mole fractions. Squares are the exact Chol superlattice mole fractions of 0.22, 0.33 and 0.50. Solid lines represent ideal mixing of mean molecular area at the designated surface pressure and were calculated using Equation 2 in Materials and Methods.

Taken together, the data in Figures 4A and 4B show that for both bovine brain and egg SM the Chol condensing effect is generally greater at lower (10mN/m) than at higher pressures (20 and 30 mN/m). For both bovine brain and egg SM mixtures at all pressures, a significant decrease in condensing effect was detected at 22.2 mol% Chol as compared to mole fractions just above and below this critical superlattice concentration.

DISCUSSION

It has been noted that the stable structure of normal myelin is the result of a delicate balance of forces to maintain its compact structure (Bradl, 1999). One important observation made in our laboratory is that whereas myelin proteins may contribute a portion of myelin intermembrane adhesion, myelin lipids also contribute to this adhesion. The contribution of myelin lipids may be critical for maintaining the balance of forces and membrane stability (Ohler et al., 2003, submitted).

Before considering the biophysical significance of the changes in the condensing effect of Chol on SM reported here, we must analyze whether the changes are indicative of myelin. This model study examined only binary mixtures of either brain or egg SM with Chol. Unfortunately myelin membranes are not this simple. It is very difficult to make a truly representative model of myelin since there exists not only a clear asymmetry in the lipid composition of the cytoplasmic and extracellular sides of the myelin membrane (Morell, 1984), but also different domains most likely exist on either sides of the bilayer. Previous studies have shown that sphingolipids (galactocerebrosides, sphingomyelin, sulfatides) reside exclusively in the extracellular leaflet due to their synthesis and shuttling mechanism from the Golgi apparatus (Lannert et al., 1998). Cholesterol is also preferentially segregated in myelin. Approximately two-thirds of the Chol mole fraction exists in the extracellular leaflet and the remaining one-third is in the cytoplasmic layer (Casper and Kirschner, 1971). This, combined with the evidence that SM and Chol tend to cluster together in domains (Brown and London, 2000), and that Chol:SM is altered in EAE,

encouraged us to study mixtures of Chol:SM in order to simulate (parts of) the extracellular leaflet of the myelin membrane.

The effects of SM acyl chain composition: egg vs. brain SM

A significantly smaller area per molecule was found at a given pressure in bovine brain compared to egg SM. This was found not only in pure SM isotherms, but also in the mixtures of Chol:SM. This is of great interest given that the acyl chains of egg SM are generally more saturated than in bovine brain SM. One would expect molecules with unsaturated acyl chains to form monolayers with increased compressibility and larger average areas per molecule at a given pressure.

In addition to possessing more saturated acyl chains, the predominant molecular species of egg SM also possess longer chains than bovine brain SM. Herein lies the most likely explanation of this paradoxical behavior (Li et al., 2000). At room temperature, SMs with short acyl chains (e.g. 12:0) are generally found in a fluid phase, whereas SMs with longer acyl chain lengths have lower in-plane compressibilities (Li et al., 2000). In general, the shorter the saturated acyl chain, the lower the temperature (T_0) at which a lipid exhibits a two-dimensional phase transition. The lower the T_0 value, the weaker the intermolecular forces that stabilize a monolayer (Li et al. 2000). The T_0 value of 18:0 SM (the predominant SM species in bovine brain SM) is 18.8˚C, whereas the T_0 value of 16:0 SM (predominant in egg SM) is 13.0˚C (Li et al. 2000). Hence, the intermolecular stabilizing forces in egg SM are generally weaker than in bovine brain SM. Two-dimensional phase transitions were not observed in this study since both egg and bovine brain SM possess a

mixture of SMs with different acyl chain lengths and the free energy of mixing lowers the surface tension and eliminates dominant transitions (Birdi, 1998).

The Superlattice model for Chol:SM monolayers

The data above show evidence that the physical properties of Chol:SM monolayers do not vary smoothly with Chol concentration, but undergo abrupt changes at certain Chol mole fractions, most notably at 22.2 mol%. This can be explained by the formation of superlattices in the monolayer. Assuming an ideal mixing model of Chol in phospholipid membranes, where the intermolecular forces between Chol/Chol, Chol/phospholipid and phospholipid/phospholipid are the same magnitude, one finds that at 22.2 mol% Chol each Chol molecule is surrounded by two circles of acyl chains (Hui, 1989). In other words, each Chol molecule has an independent boundary ring of phospholipid molecules. Therefore the properties of the monolayer would change abruptly at this specific mole ratio. Engleman and Rothman predicted that the same phenomenon would occur at a mole ratio of 1:2 (Chol:phospholipid) (Hui, 1989). In this situation, each Chol molecule is surrounded by seven acyl chains of phospholipid molecules. Above 33.3 mol% Chol, Chol molecules may touch each other. This would also cause sudden physical changes.

At a concentration of exactly 22.2 mol% a sudden decrease in the condensation effect of Chol appears to occur. This condensation effect must be due to cross-sectional area changes that occur almost exclusively in SM because Chol is a rigid, relatively inflexible molecule with a cross-sectional area that varies little with increasing surface pressure (Smaby et al., 1996). The primary effect of incorporating Chol in a SM monolayer is

reduction of trans-gauche isomerization about the carbon-carbon bonds in the liquid-expanded acyl chains of SM (Speyer et al., 1989; Morrow et al., 1995). Transient acyl kinks become diminished so that optimal van der Waals interactions can be achieved between the hydrocarbon chains and the α-surface of the steroid ring. A secondary effect of Chol incorporation may be relaxation of the long axis molecular tilt that probably occurs in the chain-ordered states of pure SM monolayers (Smaby et al., 1996).

Our results indicate that just beyond 22.2 mol% Chol, the condensation effect suddenly increases again. This could be explained by the fact that just beyond this point, the regions containing only SM molecules suddenly disappear (Jain, 1988). Suddenly, every single SM molecule is in contact with a Chol molecule and is able to experience the condensation effect (reduction of the trans-gauche isomerization in the acyl chains) of Chol. Why we also witnessed a major condensation effect just before this assumed critical lattice cannot be explained by this hypothesis. We should also consider the possibility that this may be due to experimental errors. This may also explain why our results at 33.3 and 50.0 mol% Chol were not as marked as at 22.2 mol%.

Most reports of superlattices in membranes show dramatic changes around the concentrations evaluated in the current study, whereas only 0.1-0.3 mol% away from these superlattice concentrations the membrane properties become normal again (Virtanen et al., 1998). For future isotherm studies, it would be interesting to observe the condensation effect of Chol at concentrations further away from the critical superlattice concentrations, as well as to observe the fluorescence images correlated with the current results.

Furthermore it would be interesting to repeat the current studies with purified SM molecules of one specific molecular species, for example pure 16:0 SM (the predominant species in egg SM) or pure 18:0 SM (the predominant species in bovine brain SM). Isotherms obtained with only one particular SM may show more marked phase transitions. Biological membranes, however, contain a mixture of SMs with different acyl chain compositions and the current study suggests that changes in Chol:SM, such as occur in EAE, may result in phase transitions that contribute to the demyelination process. Further studies as described above are required to determine this effect.

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