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## THE BILAYER STABILIZING ROLE OF SPHINGOMYELIN IN THE PRESENCE OF CHOLESTEROL

### A $^{31}\text{P}$ NMR STUDY

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### Summary

1. The polymorphic phase behaviour of bovine brain sphingomyelin alone and in the presence of soya phosphatidylethanolamine (which prefers the hexagonal ( $H_{II}$ ) configuration in isolation) has been investigated employing  $^{31}\text{P}$  NMR techniques. The influence of cholesterol on the phase behaviour of these systems has also been characterized.

2. The  $^{31}\text{P}$  NMR spectra obtained from dry and hydrated sphingomyelin indicate that the local motion and conformation in the phosphate group region is similar to that of glycerol-based phospholipids. In addition, in the presence of excess water the bulk of the sphingomyelin displays  $^{31}\text{P}$  NMR spectra consistent with the bilayer configuration. Further, a hydrocarbon phase transition is apparent as the temperature is increased through  $35^\circ\text{C}$ , in agreement with calorimetric results. This transition is removed by equimolar cholesterol.

3. In the absence of cholesterol, bovine brain sphingomyelin has a similar ability to stabilize the bilayer configuration when mixed with soya phosphatidylethanolamine as does 16 : 0/16 : 0 or egg yolk phosphatidylcholine, and relatively complete bilayer stabilization occurs at 30 mol% or higher sphingomyelin contents. The mixed systems containing sphingomyelin display much less tendency to adopt non-bilayer phases when the temperature is raised to  $70^\circ\text{C}$ , in contrast to their phosphatidylcholine-containing counterparts.

4. In the presence of cholesterol, 30 mol% sphingomyelin/70 mol% soya phosphatidylethanolamine dispersions display a much greater affinity for the bilayer configuration than do similar phosphatidylcholine/soya phosphatidylethanolamine systems, which tend to adopt non-bilayer configurations at low and/or equimolar cholesterol levels.

5. The results are consistent with a role of sphingomyelin *in vivo* which is primarily structural in nature, serving to stabilize bilayer structure, particularly in the presence of cholesterol. It is suggested that elevated levels of sphingomyelin which accompany early stages of atherosclerosis may reflect a response to high membrane concentrations of cholesterol, as sphingomyelin would more effectively maintain bilayer structure and therefore cellular integrity in such situations.

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## Introduction

The individual roles of the many different species of lipid found in biological membranes are poorly understood. Until recently it was generally thought that the major function of the lipid component was structural, providing an inert semi-permeable bilayer envelope with which functional proteins were associated. Observations that many lipid species found in significant concentrations in biological membranes preferentially adopt non-bilayer configurations in isolation [1–3] are, however, causing this view to be modified. In particular, the ability of certain lipid species to adopt non-bilayer structures has been employed to suggest dynamic roles for lipids in trans-bilayer ‘flip-flop’ [1], cell fusion [4], exo- and endocytosis [5] as well as facilitated transport [5] phenomena. Within the context of this ‘metamorphic mosaic’ [5] model, structural roles related to maintaining bilayer integrity may be assigned to lipids such as phosphatidylcholines, which adopt the bilayer phase in isolation and stabilize the bilayer configuration in the presence of ‘non-bilayer’ lipids [1,6]. Alternatively, lipids adopting non-bilayer phases in isolation (such as unsaturated phosphatidylethanolamines [1,2,6,7–9] as well as cardiolipin [3,10] and phosphatidic acid [11] in the presence of  $\text{Ca}^{2+}$ ) may be considered to facilitate other functional processes requiring the presence of non-bilayer lipid structures.

In this regard it is of obvious interest to determine the possible functional roles of sphingomyelin, which is a fairly ubiquitous lipid found in high concentrations in mammalian membranes such as that of the erythrocyte [12], the myelin sheath [13] and the plasma membranes of cells constituting the arterial intimal wall [14]. Two interesting features of sphingomyelin concern its apparent ability to substitute for phosphatidylcholine in the erythrocyte membrane [15] and the fact that high levels of sphingomyelin are invariably associated with high endogenous cholesterol levels [16]. In this work we have therefore compared the bilayer stabilizing capacity of bovine brain sphingomyelin with saturated and unsaturated phosphatidylcholine, by monitoring its ability to stabilize the bilayer phase in the presence of an unsaturated phosphatidylethanolamine (which prefers the hexagonal  $\text{H}_{\text{II}}$  phase in isolation) in the presence and absence of cholesterol. It should be noted that on the basis of X-ray work [25], it is known that hydrated sphingomyelin assumes the bilayer configuration in the presence or absence of cholesterol. In agreement with this finding, but in contrast to a recent  $^{31}\text{P}$  NMR study [17] we show that sphingomyelin stabilizes the bilayer arrangement of mixed lipid systems, particularly in the presence of cholesterol. These results are discussed in relation to changes in

lipid composition in arterial wall cell membranes during development of atherosclerotic lesions.

## Materials and Methods

Bovine brain sphingomyelin and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (16 : 0/16 : 0 phosphatidylcholine) were obtained from Sigma Chemical Co. and were found to be at least 99% pure as indicated by thin-layer chromatography. A polyunsaturated phosphatidylethanolamine derived from soya bean phosphatidylcholine was prepared employing the headgroup exchange capacity of phospholipase D [18]. This phosphatidylethanolamine was subsequently purified employing carboxymethyl cellulose column chromatography [18], arriving at a white compound that was again 99% pure as indicated by thin-layer chromatography. Phospholipase D was obtained from the inner leaves of Savoy cabbage employing well established procedures [19].

Samples employed for  $^{31}\text{P}$  NMR studies were prepared from an appropriate mixture of lipids in chloroform in a 10 mm NMR sample tube (50 mg total phospholipid), where the chloroform was evaporated under nitrogen and subsequent high vacuum (1–2 h). The dry lipid was then hydrated employing 0.6 ml  $^2\text{H}_2\text{O}$  (10 mM Tris-acetic acid, pH = 7.4, 2 mM EDTA, 'buffer A'), by vortex mixing. In cases where the lipid adopted the  $\text{H}_{\text{II}}$  phase a glass agitator was employed to facilitate more rapid hydration and dispersal.

$^{31}\text{P}$  NMR studies were performed on a Bruker WP-200 spectrometer equipped with temperature control and reasonably high power proton decoupling ( $\approx 5$  G). Accumulated free induction decays were obtained from up to 5000 transients employing an interpulse time of 0.5 s, an 11  $\mu\text{s}$   $90^\circ$  pulse and sweep width of up to 60 kHz.

## Results

The bilayer or non-bilayer characteristics of the hydrated lipid dispersions were detected employing  $^{31}\text{P}$  NMR techniques which, as indicated elsewhere [1–6], provide a convenient method of identifying glycerol based phospholipids in bilayer, hexagonal  $\text{H}_{\text{II}}$  or inverted micellar, cubic or rhombic configurations. An advantage of the  $^{31}\text{P}$  NMR technique is that it is straightforward to apply to both model and biological membranes. Disadvantages are implicit in the fact that the motional properties of phospholipids are detected which in turn are sensitive to the macroscopic structures adopted, and in some cases a number of potential structures could give rise to similar spectra. For example, it may be expected that phospholipids in hexagonal  $\text{H}_1$  phase would give rise to similar  $^{31}\text{P}$  NMR spectra as  $\text{H}_{\text{II}}$  phase phospholipids. In order that such techniques may be extended to sphingosine based phospholipids such as sphingomyelin two conditions must be satisfied. First, in the rigid lattice (no motion) situation the components of the chemical shift anisotropy tensor must be similar to those observed for glycerol based phospholipids such as phosphatidylcholine. That this is the case is illustrated in Fig. 1(a) where the  $^{31}\text{P}$  NMR spectra arising from anhydrous bovine brain sphingomyelin is depicted. It may be noted that this spectrum is effectively identical to previously published

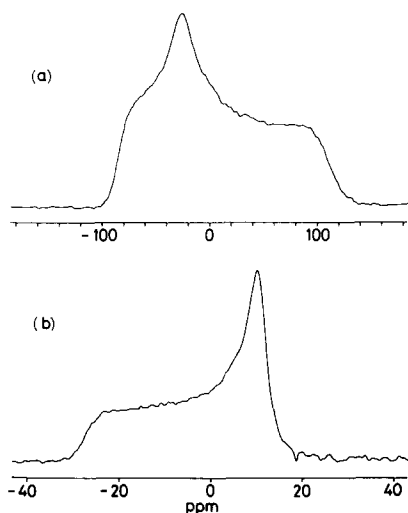


Fig. 1. 81.0 MHz  $^{31}\text{P}$  NMR spectrum of (a) anhydrous bovine brain sphingomyelin at  $20^\circ\text{C}$  and (b) partially hydrated bovine brain sphingomyelin at  $60^\circ\text{C}$ . Partial hydration was achieved by incubating 100 mg of (dry) sphingomyelin overnight at  $60^\circ\text{C}$  over a saturated salt solution. The  $\text{H}_2\text{O}$  content was then 15–20% by wt. Proton decoupling of approx. 5 G was employed for both (a) and (b), and 0 ppm corresponds to the resonance position of sonicated phosphatidylcholine vesicles in this and subsequent figures.

powder spectra of 16 : 0/16 : 0 phosphatidylcholine [20]. Similar values for the principal components of the rigid lattice chemical shift anisotropy tensor are also obtained. In short, Fig. 1a indicates similar local conformation in the phosphate group region for both sphingomyelin and phosphatidylcholine.

The second condition to be satisfied is that the local motion and conformation in the phosphate group region for hydrated liquid crystalline sphingomyelin in the bilayer configuration must be similar to that of the glycerol based phospholipids. Again, this condition is satisfied as indicated in Fig. 1b which indicates the  $^{31}\text{P}$  NMR spectra obtained from partially hydrated (20% by wt.  $\text{H}_2\text{O}$ ) sphingomyelin at  $60^\circ\text{C}$ . The partially hydrated preparation was employed as certain ambiguities arise from the spectra obtained from sphingomyelin dispersed in excess water (see below). The familiar 'bilayer' lineshape of Fig. 1b is characteristic of an axially symmetric chemical shift anisotropy tensor and gives a value of  $\Delta\sigma_{\text{CSA}}^{\text{EFF}} = -38$  ppm, typical of glycerol based phospholipids in the bilayer phase [1–6].

Fig. 2a illustrates the  $^{31}\text{P}$  NMR spectra obtained at various temperatures from sphingomyelin dispersed in an excess of the aqueous buffer. It may be immediately noted that the lineshapes at and above  $40^\circ\text{C}$  (i.e. in the liquid crystalline phase) differ from that of Fig. 1b. In particular a narrow spectral component with a chemical shift characteristic of isotropic motional averaging is apparent, which increases in amplitude as the temperature is raised. A similar spectral feature has been observed by Yeagle et al. [17], who take the composite lineshape to indicate an hexagonal ( $\text{H}_{\text{II}}$ ) arrangement of hydrated sphingomyelin. Such a possibility is not consistent with the results of Fig. 2a, however, as the narrow component does not exhibit the correct shift, occurring at a posi-

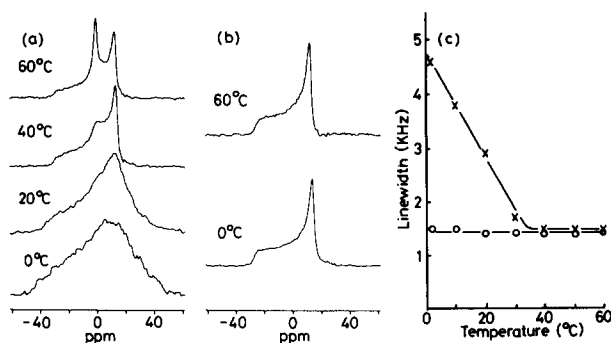


Fig. 2. Temperature dependence of  $^{31}\text{P}$  NMR spectra detected at 81 MHz of (a) bovine brain sphingomyelin dispersed in an excess of buffer A (10 mM Tris-acetic acid, pH = 7.4, 2 mM EDTA) and (b) bovine brain sphingomyelin in the presence of equimolar cholesterol. Fig. 2(c) indicates the temperature dependence of the width at half height ( $\Delta\nu_{1/2}$ ) of the  $^{31}\text{P}$  NMR spectra arising from these dispersions in the absence of broad band proton decoupling; X, bovine brain sphingomyelin; O, bovine brain sphingomyelin/cholesterol (equimolar).

tion consistent with isotropic motion (0 ppm) rather than at  $-\frac{1}{2}\Delta\sigma_{\text{CSA}}^{\text{EFF}}$  (Fig. 3). In no case, even at temperatures as high as 90°C, was any component consistent with  $\text{H}_{\text{II}}$  phase formation observed. The origin of the narrow feature is not understood, although it is of interest to note that a similar peak is often observed for saturated phosphatidylcholines (see Fig. 1c and d of Ref. 21) and possibly arises from small bilayer structures where rapid isotropic averaging due to tumbling and lateral diffusion processes occurs [22]. The important observation is that  $\text{H}_{\text{II}}$  phase formation does not occur, and that the bulk of the sphingomyelin, when in the liquid crystalline phase, contributes to a typical 'bilayer'  $^{31}\text{P}$  NMR spectrum.

The influence of a hydrocarbon phase transition is apparent in the results of Fig. 2a at 0 and 20°C, for which much broader  $^{31}\text{P}$  NMR spectra are observed. This broadening arises due to more restricted motional averaging available for phospholipids in the gel phase [21]. In the case of phosphatidylcholines the line-narrowing observed (in the absence of proton decoupling) as the temperature is raised through the gel-liquid crystalline transition temperature ( $T_c$ ) has been employed to obtain a measure of  $T_c$  [21]. Such techniques may also be extended to sphingomyelin, as indicated in Fig. 2c. By analogy with previous results [21] these data indicate  $T_c \approx 35^\circ\text{C}$ , which coincides with the midpoint of the transition detected by differential scanning calorimetry [23]. The ability of equimolar concentrations of cholesterol to eliminate this transition is also indicated in Fig. 2c by the constant width at half height of the  $^{31}\text{P}$  NMR spectra exhibited by these preparations. Finally, the  $^{31}\text{P}$  NMR spectra obtained (employing proton decoupling) from sphingomyelin in the presence of equimolar cholesterol are shown in Fig. 2(b). It may be noted that all the phospholipid contributes to a classical 'bilayer'  $^{31}\text{P}$  NMR spectrum where  $\Delta\sigma_{\text{CSA}}^{\text{EFF}} = -38$  ppm.

It therefore appears that  $^{31}\text{P}$  NMR techniques may be usefully employed to monitor the polymorphic phase behaviour of hydrated sphingomyelin and sphingomyelin-cholesterol systems, and that in the absence of other factors at

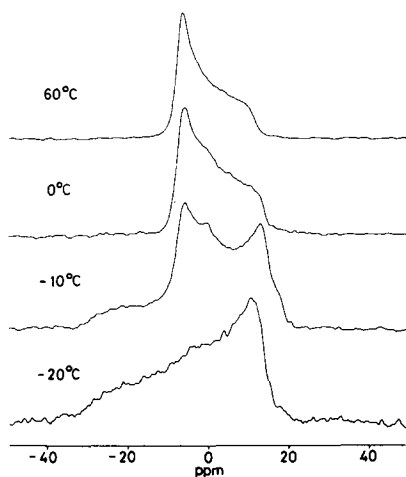


Fig. 3. Temperature dependence of the 81 MHz  $^{31}\text{P}$  NMR spectra arising from phosphatidylethanolamine derived from soya phosphatidylcholine (see Methods) dispersed in buffer A. Broad band proton decoupling was employed.

least the bulk of the sphingomyelin exhibit  $^{31}\text{P}$  NMR spectra consistent with the bilayer phase. It is then of interest to compare the strength of this predilection of sphingomyelin for the bilayer arrangement with that of other phospholipids, which may clarify possible functional roles of sphingomyelin *in vivo*. In particular, it has been noted elsewhere [1,6] that phosphatidylcholines of varying fatty acid composition prefer the bilayer arrangement in isolation, and that such lipids stabilize the bilayer phase in the presence of lipids which assume non-bilayer configurations in isolation. This leads to the conclusion that a major functional role of phosphatidylcholines is to maintain the bilayer structure of membranes, which is of course vital to cellular integrity. In addition, it has been shown that in mixtures of unsaturated phosphatidylethanolamines

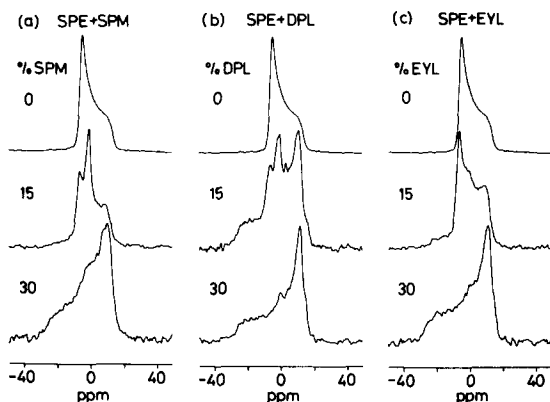


Fig. 4. Influence of increasing amounts of (a) bovine brain sphingomyelin; (b) 16 : 0/16 : 0 phosphatidylcholine and (c) egg yolk phosphatidylcholine on the 81.0 MHz  $^{31}\text{P}$  NMR spectra of soya phosphatidylethanolamine at 40°C. All systems were dispersed in buffer A and proton decoupling was employed. SPE, soya phosphatidylethanolamine; SPM, sphingomyelin; DPL, dipalmitoyl phosphatidylcholine; EYL, egg yolk phosphatidylcholine.

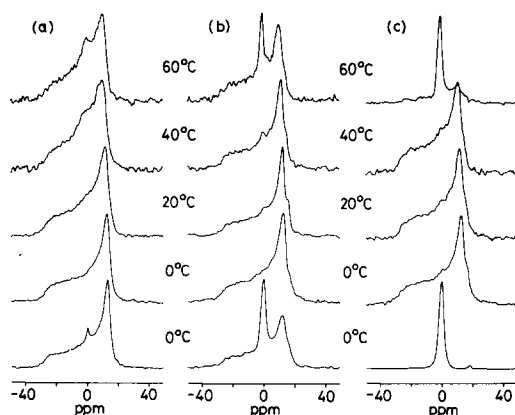


Fig. 5. Temperature dependence and hysteresis exhibited by 81.0 MHz  $^{31}\text{P}$  NMR spectra of mixtures of soya phosphatidylethanolamine with (a) 30 mol% bovine brain sphingomyelin; (b) 30 mol% 16 : 0/16 : 0 phosphatidylcholine and (c) 30 mol% egg yolk phosphatidylcholine. The lowest spectra in each case were obtained on returning to 0°C after heating the sample to 70°C for 15 min. Buffer A was employed in all cases, as was broad band proton decoupling. All percentages are expressed on a molar basis.

(which prefer the hexagonal ( $H_{II}$ ) phase in isolation) with unsaturated phosphatidylcholines cholesterol can destabilize bilayer structure [1,6]; whereas the addition of cholesterol to similar systems containing saturated phosphatidylcholines positively stabilizes the bilayer phase [1,6]. As high sphingomyelin contents are invariably associated with high endogenous cholesterol levels in vivo, the possibility exists that sphingomyelin is incorporated in preference to naturally occurring (i.e. unsaturated) phosphatidylcholines because it can maintain bilayer structure more effectively in such situations.

In order to subject this proposal to detailed examination, we investigated the bilayer stabilizing capacity of bovine brain sphingomyelin as indicated by its

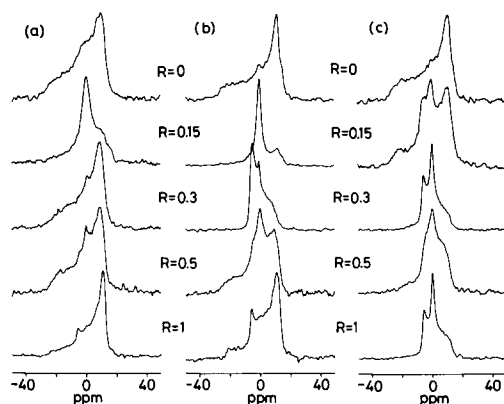


Fig. 6. Influence of increasing cholesterol content on the 81.0 MHz  $^{31}\text{P}$  NMR spectra at 40°C arising from soya phosphatidylethanolamine in the presence of (a) 30 mol% bovine brain sphingomyelin; (b) 30 mol% 16 : 0/16 : 0 phosphatidylcholine and (c) 30 mol% egg yolk phosphatidylcholine. Buffer A and broad band proton decoupling were employed throughout. R indicates the molar ratio of cholesterol to phospholipid.

ability to stabilize the bilayer configuration in the presence of 'non-bilayer' lipid and monitored the influence of cholesterol. The non-bilayer lipid chosen was a very unsaturated phosphatidylethanolamine derived from soya phosphatidylcholine. The preference of this lipid for the  $H_{II}$  arrangement at temperatures above  $0^{\circ}\text{C}$  is illustrated in Fig. 3, where a bilayer to hexagonal  $H_{II}$  transition is apparent in the region of  $-10^{\circ}\text{C}$ . Due to the similar polymorphic phase behaviour and fatty acid composition of this lipid and naturally occurring soya phosphatidylethanolamine [1] we shall hereafter refer to it as 'soya phosphatidylethanolamine'.

The ability of increasing amounts of bovine brain sphingomyelin to stabilize the bilayer phase in the presence of soya phosphatidylethanolamine (at  $40^{\circ}\text{C}$ ) is illustrated in Fig. 4a. Fig. 4b and c illustrate the effects of comparable amounts of 16 : 0/16 : 0 and egg yolk phosphatidylcholine. It may be noted that the sphingomyelin induces the bilayer arrangement at similar concentrations as do the phosphatidylcholine species, leading to the conclusion that sphingomyelin is an effective agent for stabilizing bilayer structure.

The results of Fig. 4c contrast with previous data (see Fig. 3 of Ref. 1) we have presented on the bilayer stabilizing ability of egg yolk phosphatidylcholine, where it was shown that 15 mol% egg yolk phosphatidylcholine caused all of the  $^{31}\text{P}$  NMR signal to assume a phase characterized by isotropic motional averaging. The discrepancy arises from the hysteresis exhibited by these and similar [6] lipid preparations (see Fig. 5). In previous work [1] the sample was heated to  $50^{\circ}\text{C}$  prior to obtaining data, whereas in the present work the lipid was hydrated at  $20^{\circ}\text{C}$ , and spectra were then obtained as the temperature was increased from  $0^{\circ}\text{C}$ . The temperature-dependent behaviour of soya phosphatidylethanolamine in the presence of 30 mol% sphingomyelin, 16 : 0/16 : 0 phosphatidylcholine and egg yolk phosphatidylcholine respectively is illustrated in Fig. 5a–c. It may be noted that sphingomyelin is by far the most effective agent for bilayer stabilization at higher temperatures.

The influence of varying amounts of cholesterol on the phase behaviour of soya phosphatidylethanolamine in the presence of 30 mol% sphingomyelin, 16 : 0/16 : 0 phosphatidylcholine and egg yolk phosphatidylcholine respectively is indicated in Fig. 6a–c. It is apparent that sphingomyelin is more effective in maintaining bilayer structure in these cholesterol-containing systems than either of the phosphatidylcholine species. In addition, the results of Fig. 6 indicate a most interesting behaviour as the cholesterol content is increased. In particular, low cholesterol/phospholipid ratios of 0.15 produce strong destabilization of bilayer structure, tending to induce 'isotropic' and/or  $H_{II}$  phase components. This trend is particularly apparent at cholesterol/phospholipid ratios of 0.3 for the 16 : 0/16 : 0 and egg yolk phosphatidylcholine. However, higher cholesterol contents corresponding to cholesterol/phospholipid ratios of 0.5 and 1 (equimolar) can reverse this behaviour somewhat. It is interesting to note that this reversal occurs above approximately equimolar ratios of cholesterol to the 'bilayer stabilizing' species present. It is possible that such behaviour reflects an initial association of the cholesterol with either the sphingomyelin or phosphatidylcholine components, which have been shown to exhibit a higher affinity for cholesterol than phosphatidylethanolamine [24]. These effects will be dealt with at length in a subsequent communication.



## Discussion

The major aim of this investigation was to examine the ability of sphingomyelin to stabilize bilayer structure in mixed lipid systems. The results obtained show clearly that in the absence of cholesterol sphingomyelin has approximately the same ability as saturated or unsaturated phosphatidylcholines to induce the bilayer configuration in the presence of 'non-bilayer' phospholipid. This is with the exception that bilayer systems stabilized by the presence of sphingomyelin appear to have a reduced tendency to adopt non-bilayer phases characterized by isotropic motional averaging at higher temperatures. The most important observation, however, is that in the presence of cholesterol sphingomyelin has a markedly superior ability to maintain bilayer structure than either of the two phosphatidylcholines investigated. In particular, a tendency of these mixed systems to assume the hexagonal ( $H_{II}$ ) configuration at higher cholesterol contents is inhibited, in marked contrast to the behaviour observed when sphingomyelin is replaced by saturated or unsaturated phosphatidylcholine.

We suggest that these relatively straightforward observations have potentially important ramifications for the understanding of certain progressive changes in membrane lipid composition *in vivo*. A particularly interesting example concerns changes in the lipid composition of the membranes of intimal cells constituting the arterial wall during the early stages of atherosclerosis. The available literature suggests an initial increase in endogenous cholesterol levels to a point where, in the words of Small and Shipley [14] 'for some obscure reason...(the presence of)...cholesterol appears to stimulate the compensatory synthesis of sphingomyelin or the transfer of sphingomyelin into membranes'. The results presented here could provide a rationale for such increases in endogenous sphingomyelin, in that an increased cholesterol content may threaten bilayer structure, which is of course vital to membrane, and therefore cellular, integrity. Compensatory increases in sphingomyelin content would, however, serve to relieve such problems.

In summary, the results of this investigation suggest that sphingomyelin plays a primarily structural role in biological membranes, serving to maintain bilayer structure. In comparison to phosphatidylcholine it would appear to be particularly effective in this role in membranes containing high concentrations of cholesterol.

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