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SPHINGOMYELINS IN BILAYERS AND BIOLOGICAL MEMBRANES

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Contents

I. Introduction

The major lipid components of biological membranes are phosphoglycerides, sphingolipids and cholesterol. Although considerable attention has been directed toward under-

standing the structure and properties of phosphoglycerides and cholesterol, until recently relatively little effort has been expended on sphingolipids. Sphingomyelins (*N*-acylsphingosine-1-phosphorylcholine or ceramide-1-phosphorylcholine), the simplest class of the sphingolipids, are a major lipid constituent of animal cell membranes. Thudicum first described this group of compounds in 1884, but it was not until 1927 that Pick and Bielschowsky [1] proved their structure to be *N*-acylsphingosine-1-phosphorylcholine. Fifty years later in 1962 Shapiro and Flowers [2] firmly established the fact that all sphingomyelins of biological origin are of the D-*erythro* configuration.

Naturally occurring sphingomyelins differ in the nature of the sphingosine base and in the acyl group linked to the amide nitrogen. By far the most commonly occurring base in the animal kingdom is the eighteen-carbon amine diol, 1,3-dihydroxy-2-amino-4-octadecene. This compound, called simply sphingosine, has a *trans* double bond between carbons 4 and 5. Usually present in most preparations are small amounts of the dihydro derivative, 1,3-dihydroxy-2-aminooctadecane [3–5]. Bovine kidneys have been shown to contain also phytosphingosine, 1,3,4-trihydroxy-2-aminooctadecane [6]. Other sphingosine bases have been found in only trace amounts [4–6]. The principal acyl groups found in sphingomyelins derived from most tissues, with the exception of the nervous system, are in the order of decreasing abundance palmitoyl (C16:0), nervonoyl (C24:1), C22:0 and C24:0 [7]. In brain the principal acyl group is stearoyl (C18:0). Less abundant components are C24:1 and C24:0 [8]. The acyl chain composition has been found to vary among tissues [9,10] and to be dependent upon the diet [11–13]. In addition it has been found to change with age in brain [14]. An extended discussion of the distribution of sphingomyelins is presented in Section V.

Recently, considerable work has been carried out on the metabolism and biosynthesis of this class of lipids. These studies have been reviewed in detail by Stoffel [15] and by Fredrickson and Sloan [9]. In summary, the principal biosynthetic route appears to be esterification of *N*-acylsphingosine phosphorylcholine [18,19]. Recent experiments on transformed mouse cells in culture indicate that biosynthesis may proceed via the transfer of phosphorylcholine to either sphingosine or ceramide from phosphatidylcholine [20,21]. Evidence for the existence of a similar pathway in various mouse tissues has recently been found [22]. All of the biosynthetic activity appears to be associated with the endoplasmic reticulum. The catabolism of sphingomyelins, which begins with hydrolytic cleavage to give phosphorylcholine and ceramide, has been described in some detail [23–26].

The chemical synthesis of sphingomyelin is considerably more complex than are the syntheses of phosphoglycerides. Details of the currently available synthetic routes as well as the methods of chemical characterization have been summarized by Shapiro [27].

II. Physical properties of sphingomyelins

IIA. Molecular structure

The fundamental chemical structure of sphingomyelin is shown in Fig. 1. For comparison the structure of phosphatidylcholine is also given. The numbering system used in this review, as indicated in Fig. 1, follows that suggested for ceramides by Pascher [28] and is more convenient than the system devised by Sundaralingham [29].

The molecular structures of both sphingomyelin and glycerophospholipids have as a common feature the geographical segregation of the polar and non-polar portions of the

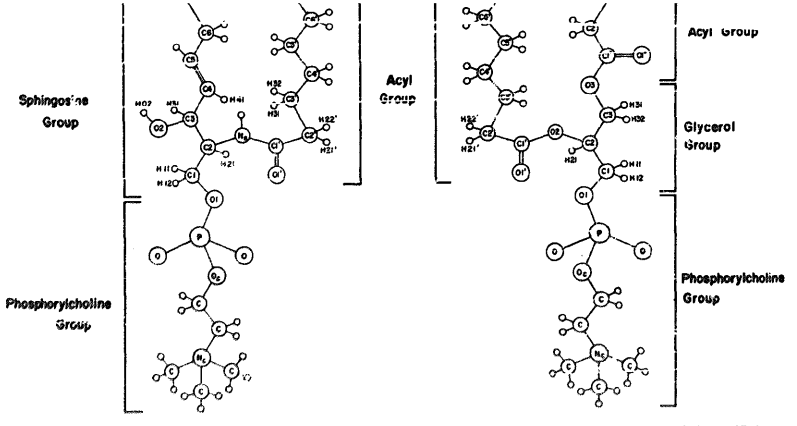

Fig. 1. The structure of sphingomyelin (left) is compared to that of phosphatidylcholine (right). The extended paraffinic groups beyond carbons 6 and 6′ in sphingomyelin and carbons 6′ and 2″ in phosphatidylcholine are not shown. The numbering is based on that suggested by Pascher [28].

molecule to give a hydrophilic head and a hydrophobic tail connected by a belt region of intermediate polarity. Because of the segregation of polarity and non-polarity within the molecule, there is no suitable solvent for both head and tail regions. As a result molecules of this type, characterized as amphipathic, aggregate to form regular though complex structures which minimize unfavorable interactions with the solvent. The structure of the aggregate depends upon the characteristics of both the amphipathic molecule and the solvent. In water, the milieu of biology, sphingomyelins and phosphatidylcholines spontaneously form bilayers when this solvent is present in excess. These lamellar structures, which are two molecules in thickness, have their opposing polar faces formed by the hydrophilic heads of the component molecules while their hydrophobic tails comprise the core of the lamella and are thus removed from the unfavorable contact with water [30,31].

Although phosphorylcholine is the polar head group common to both sphingomyelins and phosphatidylcholines, the other regions of each type of molecule have certain distinctly different structural features. The hydrophobic region of phosphatidylcholine is composed of two acyl groups esterified to the glycerol backbone. These acyl chains are almost equal in length. In naturally occurring phosphatidylcholines, the acyl group in position 1 is saturated while that in position 2 is unsaturated. This region in sphingomyelins is composed of one acyl chain which is linked through an amide bond to the primary amino group on carbon 2 of sphingosine. More than 60% of the naturally occurring sphingomyelins contain a saturated unbranched acyl chain with a length of 16 to 24 carbons. The most common unsaturated acyl chains are derived from *cis* monoenoic acids, principally nervonic (24:1). More than 50% of the acyl chains are longer than 20 carbons. The second component of the hydrophobic region of sphingomyelin is the paraffinic residue of the sphingosine base, which contributes only 13 to 15 carbon atoms to the non-polar region. Thus, the two hydrocarbon chains comprising the hydrophobic tail of sphingomyelin differ in length by more than 7 methylene residues in over 50% of the naturally occurring molecules. In addition the average number of *cis* double bonds per sphingomyelin molecule of biological origin is 0.1 to 0.35 while for phosphatidylcholine it is 1.1 to 1.5 [10,51,52].

The differences between sphingomyelin and phosphatidylcholine in the interface region are even more striking. In phosphatidylcholine this region includes carbons 1, 2 and 3 of the glycerol backbone and the components of the two ester bonds linking the acyl groups to this backbone. In contrast, this region in sphingomyelin contains the amide bond between the acyl chain and the primary amino group on carbon 2 as well as the hydroxyl group attached to carbon 3 and possibly the *trans* double bond between carbons 4 and 5 of sphingosine. The hydroxyl group and the amide bond afford an important hydrogen bond donor capability not found in phosphatidylcholine. In addition, the presence of these groups in the belt region, where a relatively low dielectric constant exists, suggests that the hydrogen bonds formed by these groups to other phospholipids, cholesterol and proteins will be stronger than hydrogen bonds formed in an aqueous medium of high dielectric constant [32,33]. Detailed molecular structure information derived from simple crystal X-ray diffraction studies is not available for sphingomyelins. Some information of this type is, however, available for the related ceramides [28,29,34]. The structural differences between sphingomyelin and phosphatidylcholine in the belt and hydrophobic regions are reflected in differences in the physical properties of these two types of lipids in bilayer systems. As is the case with phosphatidylcholines, the acyl chain composition has a profound effect on the physical properties of the system.

The physical studies discussed in the following sections are for the most part carried out on liposome dispersions of two types. These are the large, multilamellar structures first described by Hertz and Barenholz [36] and Bangham [37,38], which are heterogeneous in size, and the small unilamellar vesicles of homogeneous size originally described by Huang [39]. To a first approximation, the bilayers in these two systems exhibit similar physical properties and structure. However, recent work suggests that the small, highly curved bilayers of the vesicle system may have certain unique properties [40–45]. In addition to studies on liposome bilayers, there has been much work on monolayers at an air/water interface. Since the information derived from this type of study has an important bearing on the interpretation of bilayer data, the discussion of the physical properties of sphingomyelin lamellar systems will begin with a brief review of the monolayer work.

IIB. Studies on monomolecular films at the air/water interface

The limiting surface area for dipalmitoyl phosphatidylcholine is 43 $Å^2$ per molecule and for egg phosphatidylcholine 56 $Å^2$ per molecule [46]. Bovine brain sphingomyelin has a limiting molecular area at the air/water interface of 42–45 $Å^2$ [46,47]. However, if a bovine brain sphingomyelin fraction enriched in *N*-stearoylsphingosine phosphorylcholine is studied, the liquid condensed film has a minimal molecular surface area of only 40 $Å^2$. A fraction enriched in *N*-nervonylsphingosine phosphorylcholine exhibits a minimal area per molecule of 56 $Å^2$ in a film of the liquid-extended type. The difference in the surface properties of these two fractions is due primarily to the *cis* double bond between carbons 15 and 16 in the nervonyl sphingomyelin. Thus, if this sphingomyelin is converted to *N*-lignoceryldihydrosphingosine phosphorylcholine by hydrogenation, the limiting surface area per molecule is reduced to 40 $Å^2$, although hydrogenation of the fraction enriched in *N*-stearoylsphingosine phosphorylcholine causes no change in the area per molecule. Since hydrogenation of both fractions also converts the sphingosine moiety to dihydrosphingosine, these data indicate that the *trans* double bond between carbons 4 and 5 has little effect on the limiting area per molecule [46].

The surface potentials of films of dipalmitoyl phosphatidylcholine and bovine brain sphingomyelin determined under similar conditions are markedly different. The relatively larger surface potential of sphingomyelin monolayers is reduced by hydrogenation indicating that the *trans* double bond between carbons 4 and 5 makes a strong contribution to the observed potential [46]. Determination of Ca^{2+} binding affinity by monolayers of these two lipids using surface potential measurements indicates that this cation has a lower affinity for sphingomyelin than for phosphatidylcholine. Similar results have been obtained with other multivalent cations [48]. These differences in binding affinity may be due to an ion-dipole interaction or hydrogen bond formation between the hydroxyl group and the phosphate oxygens of sphingomyelin which cannot occur in phosphatidylcholine [46,49]. It has, however, been suggested that the observed differences in surface behavior of these two phospholipids may be due to impurities present in both preparation [50].

IIC. Thermotropic behavior

In a pioneering study of bovine brain sphingomyelin of undefined fatty acid composition, Reiss-Husson [30] using low-angle X-ray diffraction found this phospholipid prep-

aration when dispersed in water to be in a gel phase at 25°C but in a liquid crystalline lamellar phase at 40°C. At 40°C the lamellar phase incorporates a maximum of 40% water by weight with additional water forming a bulk phase. Reiss-Husson also found that the surface area per molecule and the interlamellar spacing both increase as the sphingomyelin is progressively hydrated. At the maximum hydration of 40%, the area per molecule was found to be 54 Å^2, and the lipid bilayer and water layer thickness to be 48 and 30 Å, respectively.

Recently, the phase behavior of aqueous dispersions of sphingomyelin with a well defined fatty acid composition was studied by Shipley and coworkers [31] using polarized light microscopy, differential scanning calorimetry and X-ray diffraction. Fig. 2 shows the temperature-composition phase diagram of the bovine brain sphingomyelin/water system as constructed by Shipley and coworkers [31]. Lamellar phases in which water is intercalated between sheets of lipid molecules arranged in a bilayer fashion were found to be present over much of the phase diagram. An order-disorder transition separates the high-temperature liquid crystalline lamellar phase from a more ordered lamellar phase at low temperature. The thermotropic behavior in the absence of water proved to be similar to that exhibited by various phosphatidylcholines. At 87°C, a transition occurs from a crystalline phase with the sphingomyelin organized in bilayers, to a liquid crystalline phase with a mobile lamellar structure. Formation of a viscous isotropic phase occurs at 144°C which at 170°C is transformed to give an hexagonal-type structure with the lipid head groups forming the core of rods packed with axes parallel in a regular two-dimensional hexagonal lattice. Increasing the amount of water to about 10% causes the gel-liquid crystalline transition temperature to decrease progressively to a value of about 40°C which then is independent of water concentration above 10%. At 47°C, this sphingomyelin preparation shows a maximum water uptake of 35% (w/w). Above this water content, the maximally swollen lamellar lipid phase coexists with an excess bulk water phase. At this limiting hydration, the area per molecule was found to be 60 Å^2 and the lipid bilayer and water layer thickness 38 and 22.2 Å, respectively. The differences between these values and those reported earlier by Reiss-Husson [30] may be due to differences in fatty acid composition of the sphingomyelin but are more probably due to differences in the temperature of the measurements [31]. Shipley and coworkers have pointed out that the maximum hydration of 35% found for bovine brain sphingomyelin

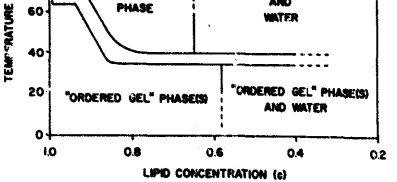

Fig. 2. The temperature-composition phase diagram of bovine brain sphingomyelin/water [31]. Reproduced by permission of the Journal of Lipid Research, Inc.

at 47°C is similar to that of egg phosphatidylcholine at 5°C. Since the two systems at these temperatures are just above the gel-liquid crystalline phase transition, these authors suggested that the phosphorylcholine head group, which is common to both, is the principal factor controlling the swelling behavior of these two types of phospholipids [31].

Below the transition temperature this sphingomyelin preparation exists in a bilayer structure which exhibits a maximum hydration of 42%. At this temperature (25°C), maximally hydrated sphingomyelin is found to have an area per molecule of 57.6 Å^2 and a bilayer thickness of 42.5 Å. The anhydrous sphingomyelin has an area per molecule of 36.1 Å^2 and a bilayer thickness of 63.5 Å at the same temperature. These data led the authors to assume a β-type structure in which the hydrocarbon chains are packed in a pseudohexagonal lattice with rotational disorder [53,54]. A structure of this type has been described for phosphatidylcholine with heterogeneous acyl chains [55]. They also suggested that the changes in the molecular packing parameters which occur with increasing hydration may be due to a progressive tilt of the hydrocarbon chain axis in a β'-type structure. This structure is known to occur in synthetic diacyl phosphatidylcholines [55].

The heat capacity vs. temperature function determined by differential scanning calorimetry for hydrated bovine brain sphingomyelin preparations shows two endothermic peaks [31,56,57]. The detailed structure of the peak system is a function of hydration. In addition, the temperatures of the two transitions as well as the temperature difference between them decrease with increasing hydration [31]. The thermodynamic data for maximally hydrated preparations of ovine and bovine brain sphingomyelin preparations as determined by Barenholz and coworkers [56] are summarized in Table I. These data were obtained with a high-sensitivity differential scanning calorimeter [45,56,58] and by drop heat capacity calorimetry [56]. Similar information on these systems has also been obtained from measurements of the depolarization of fluorescence of the probe, 1,6-diphenylhexa-1,3,5-triene [56]. Recently, Calhoun and Shipley [59] have shown that differences in the acyl chain compositions of sphingomyelins prepared from a variety of natural sources have a marked influence on the thermal behavior of the fully hydrated

TABLE I

THERMODYNAMIC PARAMETERS FOR THE GEL-LIQUID CRYSTALLINE TRANSITION OF SHEEP BRAIN AND BOVINE BRAIN SPHINGOMYELINS *

Sheep: the sets of parameters refer to three calorimetric experiments with the same preparation. 1 was freshly prepared, 2 was a repeat of 1 after a week's storage in the cold and 3 was a repeat of 2 immediately after cooling. Bovine: the two sets of parameters refer to two experiments with different liposome preparations using the same material

Species		T_{M1} (°C)	T_{M2} (°C)	T_{M3} (°C)	ΔH (kcal/mol)	$\Delta C_{p,max}$ (kcal/mol per K)
Sheep	1	31.0	37.0		7.1	0.523
	2	31.2	37.1		7.6	0.521
	3	31.3	37.1		6.8	0.516
Bovine	1	30.4	32.5	38.4	6.9	0.580
	2	(~30) **	32.4	37.0	6.6	0.550

* Data from Ref. 56.

** Shoulder observed at approx. 30°C.

systems. Thus, sphingomyelins with a relatively high content of palmitic and stearic acids have gel-liquid crystalline transitions at relatively high temperatures (about 50°C) whereas samples rich in nervonic acid (C24:1) exhibit a more complex transition at lower temperatures (about 30°C) [59].

The thermotropic behavior of aqueous liposome dispersions formed from several synthetic sphingomyelins of the DL-*erythro* configuration prepared by the synthesis of Shapiro [27] is shown in Fig. 3. The thermodynamic parameters for these synthetic preparations are given in Table II [56]. Sphingomyelins of the D-*erythro* configuration with these acyl chain and sphingosine base compositions are the major constituents of bovine and ovine brain sphingomyelins [56]. Although the synthetic preparations are racemic mixtures of D- and L-*erythro* configurations, Calhoun and Shipley [60] have recently shown that the thermotropic behavior of D-*erythro*-*N*-palmitoyl sphingomyelin [60] is very similar to that of the DL-*erythro* mixture [56].

The single sharp transition exhibited by each of these four synthetic sphingomyelins is reminiscent of the gel-liquid crystalline phase transition of synthetic phosphatidylcholines [61,62]. However, the simple linear increase in the transition enthalpy change and transition temperature with increasing acyl chain length, which holds for saturated diacyl phosphatidylcholines, is not obtained for these synthetic sphingomyelins. This is illustrated in Fig. 4 in which ΔH is plotted vs. T_m for the gel-liquid crystalline phase transition of C:12, C:14, C:16, C:18 and C:22 diacyl phosphatidylcholines (points 1–5, respectively) [192]. In contrast, points 11, 14 and 15 give the parameters characterizing C:16, C:24 and C:18 sphingomyelins, respectively. Thus, for sphingomyelins, although there is a linear relation-

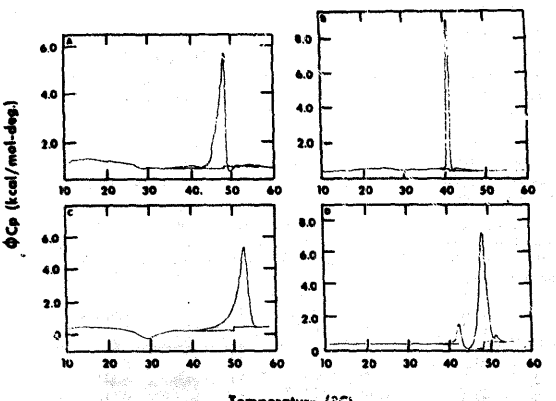

Fig. 3. Heat capacity (ϕC_p) vs. temperature for several synthetic sphingomyelin liposome preparations. (A) *N*-Palmitoyldihydrosphingosine phosphorylcholine (concentration 1.2 mg/ml); (B) *N*-palmitoylsphingosine phosphorylcholine (concentration 10 mg/ml); (C) *N*-stearoylsphingosine phosphorylcholine (concentration 10 mg/ml); (D) *N*-lignocerylsphingosine phosphorylcholine (concentration 10 mg/ml). Reproduced by permission of the American Chemical Society.

TABLE II

THERMODYNAMIC PARAMETERS FOR THE GEL-LIQUID CRYSTALLINE TRANSITION OF SOME SPHINGOMYELIN-CONTAINING LIPOSOMES *

Parameters for only the most prominent transition are reported

	T_m (°C)	ΔH (kcal/mol)	$\Delta C_{p,max}$ (kcal/mol per K)	$\Delta T_{1/2}$ (°C)
N-Palmitoyldihydrosphingosine-phosphorylcholine	47.8	9.4	4.7	1.8
N-Palmitoylsphingosine-phosphorylcholine	41.3	6.8	8.6	0.8
N-Stearoylsphingosine-phosphorylcholine	52.8 (57.0) ***	17.9 (20.0) ***	5.0 (5.7) ***	2.7 (1.4) ***
N-Lignocerylsphingosine-phosphorylcholine **	48.6	15.3	7.1	1.8
1 : 1 : 1 mixture of *N*-palmitoyl-, *N*-stearoyl-, and *N*-lignoceryl-sphingosine phosphorylcholine	39.5	6.8	1.5	2.6
2 : 1 mixture of *N*-stearoyl-sphingosine phosphoryl-choline and 1-palmitoyl-2-oleyl phosphatidylcholine	47.6	9.8	1.3	5.6

* Data from Ref. 56.

** A small transition (ΔH = 1.9 kcal/mol) is also observed at 42.6°C.

*** Data from Ref. 64.

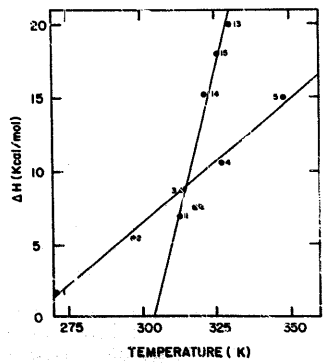

Fig. 4. Transition enthalpy change (ΔH) vs. transition temperature (T_m) for synthetic diacyl phosphatidylcholines and sphingomyelins. Datum point numbers designate compounds, 1, 2, 3, 4, 5 are for C:12, C:14, C:16 and C:22 diacyl phosphatidylcholines, respectively; 11, 12, 13, 14 and 15 are for C:16, C:18, C:18, C:24 and C:18 sphingomyelins, respectively. Points 13 and 15 are for C:18 sphingomyelin with an ordered, stable gel phase, point 12 is for this material with a metastable, gel phase. The data for phosphatidylcholines were taken from Ref. 192.

ship between ΔH and T_m, the ordering of the data does not follow increasing acyl chain length. In addition, recent work indicates that the *N*-stearoyl (C:18) sphingomyelin in bilayers exists below the transition in a form which exhibits an unusually high degree of order [64]. The transition of the gel phase of bilayers of this material to the liquid crystalline state is associated with the large ΔH and high T_m values shown in Table II (values in parentheses) and point 13, Fig. 4. If multilamellar liposomes of this sphingomyelin prepared above the transition temperature are brought quickly to 20°C and then examined in the differential scanning calorimeter, a transition at about 45°C is observed which has an enthalpy of 7 kcal · mol^{-1} (point 12, Fig. 4). The gel phase of this quenched material gives an X-ray diffraction pattern exhibiting the degree of order usually associated with bilayer systems of glycerophosphatides [64]. The transition in the gel phase from the less- to more-ordered system has a half-time of several hours at 20°C. As a result, composite patterns showing both low- and high-temperature transitions are frequently obtained. Exotherms in these patterns have also been occasionally observed. It is interesting that traces of impurities such as the fluorescent probe, 1,6-diphenylhexatriene or cholesterol cause the system to be trapped in the less-ordered form.

These differences between the thermal behavior of saturated diacyl phosphatidylcholines on the one hand and the synthetic sphingomyelins on the other are undoubtedly related to the fact that in sphingomyelins, the methylene chain contributed by the sphingosine base is of constant length in all molecular species. Thus, while in diacyl phosphatidylcholines the two methylene chains of each molecular species are always of equal length, in sphingomyelins the two methylene chains are of roughly equal length only when the acyl chain is 16 carbons long. Increasing the acyl chain length increases the length disparity between the two methylene chains of sphingomyelins (see subsection IIA). Thus, it is possible that the disparity in methylene chain length when nonexistent makes the thermal properties and degree of order of sphingomyelin and the corresponding phosphatidylcholine similar (compare points 11 and 3, Fig. 4). A larger degree of chain length disparity may force the molecules in the gel phase to assume eventually a higher degree of order and to display larger values of T_m and ΔH (compare points 12 and 13, Fig. 4). A still larger disparity in chain length, however, may cause the molecules to be trapped in a more disordered state with a resultant lowering of both ΔH and T_m (compare points 13 and 14, Fig. 4).

Mechanical coupling between the two monolayers comprising the bilayer of small unilamellar vesicles prepared from synthetic *N*-lignoceryl (C:24) sphingomyelin [191] has been demonstrated by ^{1}H-NMR. The experimental approach utilized two properties of trivalent, paramagnetic lanthanide ions: the well known fact that they can be used to separate the resonances due to molecules on the outside of small single-walled vesicles from those of molecules on the inside, and that the binding of these ions to the phosphatidylcholine head groups increases the thermotropic transition temperature. The transition was monitored using the choline methyl line-widths. These resonances give good outside/inside resolution, are of high and constant intensity through the phase transition, and show a reasonably sharp break at a temperature which corresponds well to the onset temperature of the thermotropic transition of small single-walled vesicles, as monitored by calorimetry. Data of this type obtained for *N*-lignocerylsphingomyelin clearly show that the inside monolayer onset temperature is affected by increasing the outside monolayer onset temperature. No evidence for monolayer coupling was found in experiments on *N*-stearoylsphingomyelin and dipalmitoyl phosphatidylcholine, lipids which contain methylene chains of nearly equal length. Thus, it is possible that this

coupling of the two monolayer transitions may be the result of the interdigitation of the methylene chains of the two monolayers which is favored by the marked difference in the length of the two methylene chains comprising each molecule of *N*-lignocerylsphingomyelin.

The effect of incorporation of a *trans* double bond between carbons 4 and 5 of the sphingosine moiety appears to have little effect on the character of the phase transition. For example, the difference between the T_m values for *N*-palmitoylsphingomyelin and dihydrosphingomyelin is only 6.5°C. This difference in T_m is much smaller than that observed for the effect of a *cis* double bond between carbons 9 and 10 of the acyl chain of phosphatidylcholine when either both chains are unsaturated or only the acyl chain in position 2 is unsaturated. This apparent anomaly is most likely the result of the fact that the *trans* bond in sphingomyelin is located in the belt region rather than deep in the apolar moiety. Barton and Gunstone [65] have described similar effects resulting from variation in the position of a *cis* double bond in the acyl chains in phosphatidylcholine.

Barenholz and coworkers [56] examined the thermotropic behavior of multilamellar liposomes formed from a 1 : 1 : 1 mole ratio mixture of *N*-palmitoyl-, *N*-stearoyl- and *N*-lignocerylsphingomyelins. Surprisingly, this mixture exhibits a single, rather sharp transition at a temperature below the T_m values for the individual species (Table II). With the glycerophospholipids such mixtures exhibit either a distinct transition with a T_m value lying between the T_m values of the individual components or show evidence of lateral phase separation [42,66,96]. In this particular case it appears that no phase separation occurs and thus the thermotropic behavior is a manifestation of the unique character of the mixed, but homogeneous, bilayer phase. This behavior is similar to the thermotropic behavior of bovine and ovine brain sphingomyelins, which show a transition range below the transition temperatures of the principal components of these natural mixtures [31,56]. These more complex mixtures in contrast, however, show multiple maxima in the heat capacity vs. temperature function [31,56]. Recently, Calhoun and Shipley [59] have studied the thermotropic behavior of bovine brain, egg yolk and ovine erythrocyte sphingomyelins. The differences in acyl chain composition are reflected in the thermal behavior of these preparations [59]. Clearly, much additional work needs to be done on these systems before the thermotropic behavior of complex mixtures can be understood in terms of the thermal properties and interactions of the component sphingomyelins.

IID. Molecular motions of sphingomyelin in bilayers

Information about intramolecular and whole molecule motions has been obtained for glycerophosphatide bilayers by a variety of techniques including NMR [67–70], ESR [71], fluorescence [72,73] and Raman spectroscopy [74,75]. Relatively little work of this sort has been carried out on sphingomyelin systems.

IID-1. The hydrophobic region

Mendelsohn and coworkers [75] have recorded Raman spectra at relatively high resolution for anhydrous bovine brain sphingomyelin of undetermined acyl chain composition above and below the gel-liquid crystalline transition. Using Raman data, these authors found the anhydrous material to undergo a crystalline-liquid crystalline transition at 90°C in good agreement with the results of Shipley and coworkers [31]. They also found evidence for the existence of *gauche* isomers in the gel phase. The number of *gauche* isomers was observed to increase steadily as T_m was approached. Recently, Fai-

man [76] has extended Raman studies to multilamellar liposomes formed from bovine brain sphingomyelin in excess water.

In general, the most informative regions of the Raman spectrum of sphingomyelin are the skeletal optical mode region (C-C stretching) between 1000 and 1150 cm^{-1}, and the C-H stretching region between 2800 and 3100 cm^{-1} which includes the methylene symmetric C-H stretch (approx. 2850 cm^{-1}) and the C-H asymmetric stretch (approx. 2890 cm^{-1}). Both regions are affected by the *trans-gauche* isomerisation and by intermolecular motion and disorder at higher temperatures [74,75]. The sharp methylene-twist vibration at 1298 cm^{-1} broadens considerably at the gel-liquid crystalline transition temperature probably because of intermolecular interactions [77]. Several other regions in the sphingomyelin spectrum appear sensitive to structural change although the origin of the molecular vibrations involved is unclear [75].

Bovine brain sphingomyelin of undefined fatty acid composition has been studied by Long and coworkers [78] with spin label probes. Using orientated thin films of this preparation with the cholesterol analog probe, 3-spiro-2'-(*N*-oxyl-4',4'-dimethyloxazolidine)-cholestane, these workers were able to obtain only powder spectra from dry and hydrated films. They concluded from these data that sphingomyelin does not form bilayer systems. This surprising result is in direct conflict with a variety of data obtained by other means [30,31]. The explanation was subsequently provided by Oldfield and Chapman [79] who showed that cholesterol analogs, such as the probe used by Long and coworkers [78], which do not contain a 3-hydroxy function are not incorporated to any significant extent into bilayer structures. Both Long and coworkers [78] and Oldfield and Chapman [79] found the stearic acid probe of the hydrophobic region of the bilayer, 12-spiro-2'-(*N*-oxyl-4',4'-dimethyloxazolidine)stearic acid, to be highly immobilized at 20°C in bovine brain sphingomyelin. These data were very similar to those obtained with this probe incorporated into the gel phase of dipalmitoyl phosphatidylcholine at 20°C [79].

The hydrophobic region of sphingomyelin bilayers in unsonicated liposomes has been studied by both ^{1}H-NMR [57] and ^{13}C-NMR [80]. Well defined spectra were obtained with a 220 MHz spectrometer at 60°C using bovine brain sphingomyelin of undefined fatty acid composition [57] in the form of multilamellar vesicles. The resolution of the spectra is rather surprising, since only poor-resolution proton spectra have been obtained at this frequency for multilamellar systems formed from glycerophosphatides at this temperature [80,81]. At 40°C, which is the center of the broad gel-liquid crystalline phase transition exhibited by this preparation, there was a decrease in intensity as well as a broadening of the signals from the acyl chain methylene and methyl protons. It seems clear that with the 5000 Hz sweep-width employed in these studies, signals were obtained only from regions in the liquid crystalline state. At 20°C, well below the phase transition range, no acyl chain signal was detected.

High-resolution proton spectra have been reported by Barenholz and coworkers [82] for bovine brain sphingomyelin of known acyl chain composition in small vesicle dispersions. In general, the spectra are similar to those obtained for small vesicles of dipalmitoyl phosphatidylcholine [43]. In the sphingomyelin systems at 21°C the methylene linewidth is 47 Hz. This decreases to 22 Hz at 52°C. At 24°C, the intensity of the methylene peak relative to the *N*-methyl proton peak is only 5% of the intensity at 50°C. The temperature course of the line-width change clearly reflects the gel-liquid crystalline phase transition discussed in the preceding section. T_1 values obtained for the methylene protons are 0.29 ± 0.03 s at 29°C and 0.395 ± 0.03 s at 52°C. These are smaller than the corresponding values obtained for egg phosphatidylcholine under similar conditions.

Arrhenius plots of T_1 vs. (temperature)$^{-1}$ gave a value of 3.4 kcal · mol^{-1} for the activation energy. This value is very similar to the values obtained for a variety of phosphatidylcholines [83,84]. In addition, the activation energy is close to the values obtained by Raman spectroscopy for internal *gauche-trans* isomerizations in phosphatidylcholine [85,86].

^{13}C-NMR spectra obtained from unsonicated multilamellar liposomes formed from bovine brain sphingomyelin were first reported by Keough and coworkers [80]. The resolution at 25.2 MHz and 70°C proved to be very good and a number of resonances were identified. Chemical shifts measured relative to an external tetramethylsilane standard are: 30.7 ppm for the broad methylene group, 23.1 ppm for the penultimate methylene and 14.4 ppm for the terminal methyl group. A broad signal at 131.2 ppm was identified with the *cis* double bond carbons of the acyl chain. The carbonyl carbon was clearly visible at 177.2 ppm and signals at 67.4, 60.8 and 55.0 ppm were identified with the CH_2^5N, CH_2OP and $N^+(CH_3)$ carbons, respectively, of the phosphorylcholine head group. While these chemical shifts are similar to the corresponding shifts observed for egg phosphatidylcholine systems [80], there are some significant differences. Both the carbonyl and unsaturated carbon resonances are shifted downfield in sphingomyelin (3.7 and 3.2 ppm, respectively). Keough and coworkers suggested that the carbonyl shift is due to the nitrogen of the amide bond whereas the shift of the unsaturated carbons may be due to the deshielding by the vicinal hydroxyl group in the sphingosine moiety [80]. Recent work, however, on synthetic *N*-stearoyl- and *N*-nervonoylsphingomyelins and on bovine brain sphingomyelin in small vesicle dispersions indicates that the acyl chain carbons of the *cis* double bonds give rise to very much sharper signals than do carbons 4 and 5 of the *trans* double bond in the sphingosine moiety (Schmidt, C., unpublished observations). Thus, in all probability the line in the sphingomyelin spectrum assigned to the double bond carbons by Keough and coworkers [80] is in fact due principally to only the acyl chain double bond carbons.

The apparent fluidity of the hydrophobic region of bilayers formed from bovine brain sphingomyelin of well defined composition has been determined by fluorescence polarization studies using as a probe 1,6-diphenylhexa-1,3,5-triene [73,88,89]. The rotational motion of the probe molecule deduced from the fluorescence polarization data is characterized by the anisotropy parameter [89,90]. The temperature variation of this parameter can be used with the Perrin equation to define an apparent microviscosity as seen by the probe molecule [73,91,92]. The microviscosity thus defined can be used in a relative sense to characterize the hydrophobic core of the bilayer. Its utility when applied to bilayer and membrane systems has recently been reviewed in detail [73].

The microviscosity of the hydrophobic region of bovine brain sphingomyelin below the gel-liquid crystalline phase transition in both multilamellar liposomes and small, single-walled vesicles is about 10 times larger than the corresponding value for egg phosphatidylcholine. Above the sphingomyelin phase transition, the apparent microviscosity is about 3 times the value obtained for egg phosphatidylcholine at the same temperature [56,89]. Similar results have been obtained on these systems using perylene as the fluorescent probe [73].

Using diphenylhexatriene, bovine brain sphingomyelin exhibits a marked change in apparent microviscosity over the temperature range of the gel-liquid crystalline phase transition. The midpoint of the transition determined by fluorescence depolarization is 32.5°C, in good agreement with the calorimetrically determined transition temperature given in Table I [56,89].

IID-2. The interface region

The interface region in bilayer systems, located between the hydrophilic surface and the hydrophobic core, is the most difficult to examine specifically. There are no simple means to attach covalently fluorescent or spin probes. Proton NMR signals from this region of the constituent molecules are not as well resolved as are signals from the hydrophobic and polar head group regions. Potentially, ^{13}C-NMR affords one of the best methods of examining the interface region in bilayer systems.

Keough and coworkers [80] have demonstrated that the ^{13}C-NMR signal from the carbonyl carbon of the acyl chain is shifted to 177.2 ppm relative to an external trimethylsilane standard in multilamellar liposomes. This is 3.7 ppm farther downfield than is the corresponding carbonyl carbon signal from egg phosphatidylcholine [80]. With small unilamellar vesicles prepared from bovine brain sphingomyelin, the chemical shift of the natural-abundance ^{13}C carbonyl carbon is 175.4 ppm for the oustide whereas the similar vesicles prepared from egg phosphatidylcholine signals from the two carbonyl carbons are resolvable at 174.3 and 174.0 ppm outside and inside, respectively. The α-carbonyl gives rise to the upfield resonance [93]. Keough and coworkers have suggested that the amide bond nitrogen adjacent to the sphingomyelin carbonyl carbon is responsible for the larger chemical shift in this molecule compared to phosphatidylcholine. This explanation may, however, not be completely correct since in small vesicle systems the admixture of other phospholipids does not cause the sphingomyelin carbonyl carbon resonance to move upfield (Schmidt, C., Barenholz, Y. and Thompson, T.E., unpublished observations). This observation suggests that sphingomyelin-sphingomyelin interactions may be at least in part responsible for the increased downfield shift. This view is strengthened by the observation that the chemical shifts for the carbonyl carbons in the two lipids are nearly equal when they are dissolved in organic solvents (Schmidt, C., unpublished observation).

Natural sphingomyelins, which all have the D-*erythro* configuration of carbons 2 and 3, are optically active. The circular dichroic spectrum of bovine brain sphingomyelin in organic solvents [94,95], in aqueous dispersions of singlelamellar vesicles [94] and in low-density lipoprotein [95] shows a strong negative Cotton effect below 200 nm. The position and magnitude of the effect are dependent on the state of aggregation of the sphingomyelin molecules [94,95]. This fact is illustrated by the data in Table III. The Cotton effect is almost certainly due to the $\pi \rightarrow \pi^*$ transition of the amide group joining carbon 2 of the sphingosine moiety to the acyl chain (see Fig. 1). It may eventually prove possible to relate the positions and magnitude of the effect to the structure of the interface region in small unilamellar vesicles.

The contribution made by sphingomyelin to the circular dichroic spectra of biological membranes has largely gone unrecognized. Most workers have attributed the details of the spectra wholly to membrane proteins. The contribution by sphingomyelin in human erythrocyte ghosts is, however, about 20% below 200 nm. Failure to recognize this contribution can lead to a marked overestimate of the random-coil content of proteins in biological membranes with a high sphingomyelin content [94].

IID-3. The headgroup region

There has been considerable controversy concerning the orientation and motions of the polar head group in phosphatidylcholine. The weight of evidence favors the view that in the gel phase the axis of the phosphatidylcholine moiety is normal to the axis of the acyl chain system and in the liquid crystalline phase it is roughly parallel to the chain axis

TABLE III

MOLECULAR ELLIPTICITY OF SPHINGOMYELIN *

Sample	Physical state	$[\theta^\circ]$ (deg · cm^{-1} · dmol^{-1})	λ_0 (nm)
Sphingomyelin	vesicles	−56 000	198
Sphingomyelin/phosphatidylcholine (50 : 50)	vesicles	−54 000	198.5
Sphingomyelin/dicetyl phosphate (100 : 5)	vesicles	−51 900	197.5
Sphingomyelin/phosphatidylcholine/dicetyl phosphate (50 : 50 : 5)	vesicles	−57 900	197.5
Sphingomyelin	micelles in 20% methanol	−58 400	197
Spingomyelin	micelles in trifluoroethanol	−77 700	194

* Data from Ref. 94.

[96]. Recently, ^{31}P-NMR has been employed to investigate the conformation and motions of the polar head group of various phosphatidylcholines. With egg phosphatidylcholine in small single lamellar vesicles, a ^{31}P(^{1}H) nuclear Overhauser effect has been observed [97]. The simplest motional model which is compatable with the ^{31}P-NMR data is rotation of the phosphorus about an axis normal to the bilayer surface. During rotation the -N^{+}(CH$_3$)$_3$ protons interact intermolecularly with a neighboring phosphate group at an average angle of 90°. A correlation time of $1.4 \cdot 10^{-9}$ s has been calculated for the time constant of the ^{1}H-^{31}P interactions in the plane parallel to the bilayer surface [97]. Although in the gel phase there is considerable head group motion [98], there is a marked increase in the mobility of this group in the phase transition region. This can be seen as a linewidth decrease in the NMR signal from the -N(CH$_3$)$_3$ protons and in the values of the spin-lattice relation time, T_1, for these protons [67,83]. It can also be observed in the T_1 data obtained from ^{13}C-NMR [98,99].

The most clear-cut differences between sphingomyelin and phosphatidylcholine are detected by NMR and occur in data obtained for the choline methyl and the phosphorus resonances. The linewidth of the -N^{+}(CH$_3$)$_3$ protons decreases markedly with temperature in the phase transition region of bovine brain sphingomyelin although the intensity remains constant [82]. T_1 values for the *N*-methyl protons and ^{31}P also smaller than corresponding quantities obtained for various phosphatidylcholines [82,83,100]. The ^{31}P resonance from sphingomyelin in small single-walled vesicles is shifted 0.6 ppm downfield from that of egg phosphatidylcholine at the same temperature. This shift increases to 0.7 ppm in methanol and 1.4 ppm in chloroform. The downfield shift of the ^{31}P resonance by Pr^{3+} is about 1.2 times that observed for egg phosphatidylcholine under similar conditions. An enhanced Pr^{3+} shift of the *N*-methyl choline protons and ^{31}P in sphingomyelin has also been observed [82]. These results can be explained by the presence of an intramolecular hydrogen bond between the phosphate and either the amide or hydroxyl groups of sphingomyelin which results in a greater restriction to motion of the head group in sphingomyelin than in phosphatidylcholine [82,101].

Studies by Yeagle and coworkers [87] using $^{31}P(^{1}H)$ nuclear Overhauser effect (NOE) offer further support fot the existence of an intramolecular hydrogen bond in sphingomyelin. The authors have suggested that the larger ratio, $(NOE)_{H_2O}/(NOE)_{2_{H_2O}}$ obtained for sphingomyelin vesicles than for vesicles formed from phosphatidylcholine is due to hydrogen bonding of either the amide or hydroxyl protons to the phosphate group of the same molecule [87]. Phosphatidylcholine, of course, does not have such protons.

III. Interactions of sphingomyelin with other lipids

IIIA. Phosphatidylcholine

Untract and Shipley [102] have carried out a thorough study of mixtures of egg phosphatidylcholine and bovine brain sphingomyelin of well characterized acyl chain composition. A complete ternary phase diagram has been constructed over the temperature range 10–44°C by these workers based on data acquired by X-ray diffraction, differential scanning calorimetry and polarized light microscopy. The phase diagram shows that at 37°C in excess water, phosphatidylcholine and sphingomyelin are completely miscible and are ordered in a liquid crystalline bilayer phase. Lateral phase separation, however, does occur upon cooling to 20°C in systems containing greater than 33 mol% sphingomyelin. Under these conditions, the system is composed of a sphingomyelin lamellar gel phase and a liquid crystalline phase containing both sphingomyelin and phosphatidylcholine. Below 20°C the sphingomyelin gel phase coexists with an ordered bilayer phase composed of a stoichiometric compound containing 2 mol of phosphatidylcholine per mol of sphingomyelin. This compound can be separated from a liquid crystalline bilayer phase composed largely of phosphatidylcholine in systems containing less than 33 mol% sphingomyelin by lowering the temperature below 20°C [102].

Limited calorimetric studies have been carried out on multilamellar liposomes formed from mixtures of synthetic *N*-stearoylsphingomyelin and 1-palmitoyl-2-oleyl phosphatidylcholine [56]. The heat capacity function for a mixture of these components containing 66 mol% sphingomyelin exhibits a single asymmetric transition with a temperature maximum at 47.6°C and $\Delta H = 9.8$ kcal · mol^{-1}. The width of the transition curve at half-height is about 5.5°C. Comparison of these thermal characteristics with those of the pure sphingomyelin shown in Table II suggests that below the transition region a gel phase composed essentially of sphingomyelin coexists with a phosphatidylcholine liquid crystalline phase [56]. The heat capacity vs. temperature function for a 1 : 1 mole ratio mixture of these two compounds is considerably more complex with three poorly resolved maxima observable in the broad transition range from 10 to 40°C [56]. The thermal diagrams obtained for these two synthetic sphingomyelin/phosphatidylcholine mixtures are in quantitative agreement with the phase diagram for mixtures of egg phosphatidylcholine and bovine brain sphingomyelin. This may not be particularly surprising since the two synthetic phospholipids are major components in the two phospholipids prepared from natural sources [102].

The phase behavior of mixtures of egg phosphatidylcholine with either bovine spinal chord [103] or brain sphingomyelin (Barenholz, Y. and Thompson, T.E., unpublished observations) has been studied using the fluorescence polarization of the probe 1,6-diphenylhexa-1,3,5-triene. Systems of either small single-walled vesicles or multilamellar liposomes exhibit a broad phase transition in the range from 20 to 40°C at mole fractions of sphingomyelin in excess of about 45 mol%. The apparent microviscosity in all systems examined at all temperatures in the range from 0 to 60°C increases with increasing

content of sphingomyelin. These data are compatible with the phase diagram for the closely similar system constructed by Untracht and Shipley [102].

The *N*-methyl proton NMR resonance for bovine brain sphingomyelin mixed with N-(C^2H_3)$_3$ egg phosphatidylcholine in equimolar mixtures has been studied in small single-walled vesicles [82]. In vesicles prepared from sphingomyelin alone, the choline methyl proton signal is composed of two distinct but overlapping peaks arising from molecules on the inside and outside surfaces of the vesicle bilayer. At 100 MHz and 52°C, the splitting is 3.5 ppm and increases with decreasing temperature [82]. A similar splitting is observed in the 1 : 1 mole ratio system. It is interesting that the line-width is markedly broader in the mixture at 29°C than it is in pure sphingomyelin at temperatures at which the microviscosity is the same. This observation suggests that in the mixture the motion of the *N*-methyl system is restricted. This may be the result of interactions between the two types of phospholipids in the mixed system [82]. On the other hand, evidence for intramolecular hydrogen bonding between the sphingomyelin phosphorus and either the amide or hydroxyl hydrogen is obtained from the observation that T_1 values for the sphingomyelin *N*-methyl protons are little affected by the addition of N-(C^2H_3)$_3$ egg phosphatidylcholine up to concentrations as high as 67 mol% [82].

The fact that the intermolecular interactions between like molecules are different from those between unlike molecules in mixed sphingomyelin/phosphatidylcholine systems is illustrated by the asymmetric distribution of these lipids in the bilayer of small vesicles. ^{31}P-NMR studies of systems of this type comprised of bovine brain sphingomyelin and either egg phosphatidylcholine or dipalmitoyl phosphatidylcholine at 1 : 1 mole ratio have shown that the sphingomyelin is more concentrated on the outer surface and the phosphatidylcholine on the inner surface [82,100,105,106].

IIIB. Cholesterol

Cholesterol or other related sterols are found as components of biological membranes in all forms of organisms with the exception of a few types of micro-organisms [107,108]. There is general agreement with the interaction of cholesterol with glycerophospholipids in bilayer systems requires the β-hydroxyl group of the sterol and the carbonyl functions of the phospholipid [33,109]. This important subject has recently been reviewed by Demel and de Kruijff [190]. Much less attention has been devoted to the examination of the interactions between cholesterol and sphingolipids, in particular sphingomyelin. A number of years ago, Vandenheuval [111] suggested on theoretical grounds that molecular configurations and van der Waal's interactions should lead to a stable complex between cholesterol and sphingomyelin. It has been known for some time that there is a strong positive correlation between the contents of cholesterol and sphingomyelin in the membranes of many mammalian cells [112].

Early ^{1}H-NMR and ESR probe studies [57,79] showed that in multilamellar liposomes formed from cholesterol and bovine brain sphingomyelin the effect of the added cholesterol was to fluidize the bilayer below the phase transition and to make it less fluid above the transition temperature. Thus, at low mole fractions, the effect of cholesterol on the apparent viscosity of sphingomyelin bilayers is parallel to that observed in glycerophospholipid systems [83]. It has also been known for some time that no gel-liquid crystalline phase transition is observed in sphingomyelin systems containing more than about 40 mol% cholesterol [57,113] in marked similarity to the behavior of cholesterol-containing bilayers formed from saturated phosphatidylcholines [114,115]. More recent ^{1}H-NMR

studies on small unilamellar vesicles formed from mixtures of cholesterol and sphingomyelin have given similar results [82]. Thus, in systems containing 40 mol% cholesterol at 52°C, the line-widths of the methyl and methylene protons of the sphingomyelin are increased by 50% and the T_1 values for these protons are markedly reduced [82].

Recently, the thermotropic behavior of aqueous dispersions of multilamellar liposomes formed from mixtures of synthetic *N*-palmitoyl- or *N*-lignocerylsphingomyelin and cholesterol have been examined in detail by differential scanning calorimetry [116]. In systems containing less than 25 mol% cholesterol, the heat capacity vs. temperature function exhibits overlapping sharp and broad components. The sharp-component enthalpy decreases with increasing mole percent cholesterol and reaches a value of zero between 25 and 30 mol% cholesterol. The broad-component enthalpy maximizes at 3 to 4 kcal/mol between 10 and 20 mol% cholesterol and decreases as the cholesterol content is either increased or decreased from this range [116]. These data have been interpreted as evidence that these mixtures undergo phase separation, with the sharper endotherm corresponding to a gel-to-liquid crystalline transition of a phase enriched in sphingomyelin and the broader component associated in some manner with a phase enriched in cholesterol. Possibly, the broad component arises from a transition in the boundary region between the two phases [116]. This interpretation is based on the similarities between these systems and the well studied systems formed from mixtures of cholesterol and dipalmitoyl phosphatidylcholine [115]. Thus, there is evidence that a phase composed of a stoichiometric complex of cholesterol and sphingomyelin exists in the synthetic sphingomyelin systems similar to that shown to form in cholesterol/dipalmitoyl phosphatidylcholine bilayers [114,115].

Further evidence supporting the existence of strong interactions between cholesterol and sphingomyelin has been presented by Demel and coworkers [110] and by van Dijck [117]. These investigators have presented calorimetric evidence which suggests that there may be a preferential affinity for cholesterol shown by bovine brain sphingomyelin in ternary mixtures with either phosphatidylcholines or ethanolamines in which phase separation of the phospholipids occurs. The key observation leading to this suggestion is the fact that the inclusion of cholesterol in such mixtures results in a selective decrease in the enthalpy change of the gel-to-liquid crystalline phase transition of the sphingomyelin-rich phase as opposed to that arising from the phase composed principally of the other phospholipid [110]. These investigators suggest that this observation is evidence for the existence of a cholesterol-sphingomyelin complex in the liquid crystalline phase. This conclusion has been questioned by Calhoun and Shipley [60] who have carried out a similar calorimetric study in the ternary system: cholesterol/dimyristoyl phosphatidylcholine/*N*-palmitoylsphingomyelin. The two phospholipid components in this system are completely miscible in both gel and liquid crystalline phases and thus no lateral phase separation occurs. In this system there is no evidence for the preferential interaction of cholesterol with either component [60]. Thus, although cholesterol does appear to interact preferentially with sphingomyelin in a laterally phase-separated gel phase of sphingomyelin, it does not seem to show a preferential interaction in a molecularly mixed system such as the dimyristoyl phosphatidylcholine/*N*-palmitoylsphingomyelin in either gel or liquid crystalline configurations. This result suggests that cholesterol does not interact preferentially with sphingomyelin in the liquid crystalline states of the systems studied by Demel and coworkers [110].

Additional support for the preferential interaction between cholesterol and sphingomyelin comes from an entirely different approach. Extraction of cholesterol from cells is

more rapid and complete when delipidated serum reconstituted with sphingomyelin rather than other phospholipids is used as an extractant [118].

IV. Interaction of sphingomyelin with proteins

Although the phosphorylcholine moieties of sphingomyelin and phosphatidylcholine are identical, many enzymes which hydrolyze this group are specific for either sphingomyelin or phosphatidylcholine [23–25,120,121]. This hydrolytic activity appears to be optimal in the phase transition region [119]. In addition to the interaction of phospholipases specific for sphingomyelin, there are a limited number of systems in which the interaction of a membrane protein appears to be specifically with sphingomyelin. Kramer and coworkers [122] have shown in comparative reconstitution experiments that the proteins of the sheep erythrocyte bind more strongly to sphingomyelin than do the proteins of the human erythrocyte. This preferential binding correlates with the unusually high sphingomyelin content of the sheep erythrocyte membrane shown in Table IV [10]. Widnell and Unkeless [123,124] have reported that the 5′-nucleotidase from the plasma membranes of rat liver cells is isolated as a lipoprotein complex. The associated lipid is entirely sphingomyelin in a ratio of about 100 mol of lipid per mol of protein. Removal of sphingomyelin inactivates the enzyme [123,124]. Evans and Gurd [125] have, however, reported the purification of a similar enzyme from mouse liver plasma membranes which is active in the absence of any lipid. In spite of this report, the work of Widnell and Unkeless does suggest a preferential binding of the 5′-nucleotidase to sphingomyelin. Sandermann [126] has discussed in detail the problems of establishing specific lipid requirements for membrane-bound enzymes. The $(Na^+ + K^+)$-ATPase isolated from rabbit kidney by Lubrol extraction binds strongly to liposomes made from sphingomyelin but not to dispersions of phosphatidylcholine unless the positively charged amphiphile, stearylamine, is added [127]. Recently, it has been found that the hemolytic toxin isolated from the sea anemone, *Stoichactis helianthus*, is strongly bound to aqueous dispersions of sphingomyelin but not to liposomes formed from other erythrocyte lipids. This

TABLE IV

PHOSPHOLIPID DISTRIBUTION IN ERYTHROCYTES FROM VARIOUS MAMMALIAN SPECIES *

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; Sph, sphingomyelin; LPC, lysophosphatidylcholine; X, unidentified; n.d., not determined; n.r., not recorded

	Rat	Rabbit	Pig	Dog	Horse	Sheep	Cow	Goat	Cat	Guinea-pig	Human
PC	47.5	33.9	23.3	46.9	42.4	n.d.	n.d.	n.d.	30.5	41.1	35.7
PE	21.5	31.9	29.7	22.4	24.3	26.2	29.1	27.9	22.2	24.6	24.7
PS	10.8	12.2	17.6	15.4	18.0	14.1	19.3	20.8	13.2	16.8	13.8
PI	3.5	1.6	1.8	2.2	<0.3	2.9	3.7	4.6	7.4	2.4	5.8
PA	<0.3	1.6	<0.3	0.5	<0.3	<0.3	<0.3	<0.3	0.8	4.2	n.r.
Sph	12.8	19.0	26.5	10.8	13.5	51.0	46.2	45.9	26.1	11.1	24.7
LPC	3.8	<0.3	0.9	1.8	1.7	n.d.	n.d.	n.d.	<0.3	<0.3	n.r.
X						4.8	1.7	0.8			
Sph/PC	0.27	0.56	1.13	0.23	0.32	12.0	12.0	13.0	0.855	0.27	0.64

* Data from Ref. 10.

observation suggests that the site of binding of this cytolytic glycoprotein of molecular weight 16 000 is plasma membrane sphingomyelin [128].

Perhaps the most interesting interaction between sphingomyelin and a membrane protein is that with acetylcholinesterase [129]. This molecule, when isolated from the electric tissue of *Electrophorus electricus*, appears in electron micrographs to be clusters of 4, 8 and 12 subunits attached to a 50 nm tail which is similar in amino acid composition to collagen [130]. The tail-piece may be removed from this assembly by treatment with trypsin or collagenase [130,131]. Treatment of intact electric tissues with these enzymes leads to solubilization of acetylcholinesterase activity [132,133]: thus the implication that the collagen-like tail-piece serves to anchor the enzyme assembly to the plasma membranes of the cells in the electric organ [129]. Watkins and coworkers [129], using a flotation-type assay, have shown that purified acetylcholinesterase binds strongly at both high- and low-ionic strengths to liposomes made from bovine brain sphingomyelin. Binding to liposomes made from egg phosphatidylcholine does not, however, occur. Furthermore, binding to sphingomyelin liposomes is reduced by the inclusion of cholesterol or phosphatidylcholine in the liposomes (Cohen, R. and Barenholz, Y., unpublished observations). The observation that collagenase-treated enzyme does not bind to sphingomyelin liposomes localizes the site of interaction in the collagen-like tail of the enzyme assembly [129]. Although the molecular basis for the interaction between the collagen-like tail and the sphingomyelin bilayer system has not been established, it seems possible that the apparent specificity may rest in the ability of sphingomyelin to form hydrogen bonds with the hydroxyprolyl and hydroxylysyl residues of the tail (see subsection IIA).

In addition to being an important component of many biological membranes, sphingomyelin is also found in the circulating plasma lipoproteins of higher animals. In humans the ratio of phosphatidylcholine to sphingomyelin is about 3 : 1 in the total plasma lipoprotein fraction [134]. The ^{31}P-NMR resonances arising from each of these phospholipids can be resolved in both native and reconstituted human lipoprotein [135]. The chemical shift difference between the high-field signal from phosphatidylcholine and the downfield resonance from sphingomyelin is similar to that observed in vesicles formed from mixtures of these phospholipids [63,82,105]. The spin-lattice relaxation time for the sphingomyelin ^{31}P of 1.73 s is considerably smaller than the value of 2.3 s obtained for the phosphatidylcholine signal. Similar results have been obtained in mixed vesicle systems [97,100]. The similarity between the environments of these two phospholipids in both reconstituted lipoproteins and small vesicles has been also demonstrated by ^{13}C-NMR spectroscopy of phospholipids enriched in the *N*-methyl system [136,137]. These results suggest that the interactions between these phospholipids and the apolipoproteins do not involve the phosphorylcholine moiety of either molecule. In reconstitution experiments, sphingomyelin appears to bind strongly to apolipoprotein A-II but not to A-I which interacts preferentially with phosphatidylcholine [139]. Apolipoprotein A-II, a disulfide-linked dimer of two identical 77 amino acid polypeptides, constitutes about 30% of the protein of human plasma high-density lipoprotein [134].

V. Sphingomyelin in biological systems

VA. Distribution

Sphingomyelin is one of the major lipid components of the cellular membranes of mammals [51]. It is also an important component of the serum lipoproteins [140,141]. Membranes from diverse sources exhibit a wide range of sphingomyelin compositions. In

many systems, however, the sum of the two choline lipids, sphingomyelin and phosphatidylcholine, constitute about half of the total phospholipid, although the ratio of the amounts of these two components varies greatly [10,51].

Large differences in the sphingomyelin-to-phosphatidylcholine ratio exist among the membranes of the various organs and tissues of a single mammalian species. For example, in the case of the cow the ratio varies in the following order: lung > spleen > kidney > brain > liver [10]. It is interesting, however, that with the exception of brain only minor variations in the ratio are observed when the same organs from different mammalian species are compared [10]. Large variations in the ratio exist among the brain tissue of various species [52]. In an excellent review, Rouser and coworkers [52] have pointed out that within a single brain, lipid-rich regions have higher sphingomyelin-to-phosphatidylcholine ratios than do lipid-poor regions. In spinal cord, the ratio is even higher than in brain [142].

In the case of the erythrocyte membrane, one of the most widely studied systems [143], the sphingomyelin-to-phosphatidylcholine ratio varies with mammalian species from 0.25 in rats to above 12 in ruminants [10,142]. Data for 11 species are summarized in Table IV. In cells with a full complement of subcellular organelles, there is within these membrane systems a variation of the sphingomyelin-to-phosphatidylcholine ratio. The ratio is highest in the plasma membrane and lowest in both nuclear and mitochondrial membranes. The endoplasmic reticulum and Golgi membranes show intermediate values [10,51,112]. Based on the morphology of the generalized cell, there is thus an increasing gradient in the ratio from the cell center to the periphery [52]. The positive correlation between the sphingomyelin and cholesterol content of many membrane systems in mammals has already been mentioned in subsection IIIB. This correlation fails, however, in erythrocytes where the cholesterol-to-total phospholipid ratio is constant [51].

There is considerable evidence to suggest that there is a marked asymmetric distribution of lipids between the outer and inner faces of several plasma membranes or plasma membrane derivatives [144]. The best documented system is the membrane of the human erythrocyte. Data obtained in experiments utilizing phospholipases [120,142–147], phospholipid exchange proteins [148] or chemical labeling reagents [149–151] clearly show that essentially all of the sphingomyelin and most of the phosphatidylcholine are located in the external surface of this membrane. Phosphatidylethanolamine and phosphatidylserine are the major lipid constituents of the cytoplasmic surface. Associated with this transmembrane lipid compositional asymmetry is an absolute compositional asymmetry of protein components [142,149,151]. A similar distribution of sphingomyelin has been demonstrated in rat erythrocytes [152], ovine erythrocytes [153], LM cell plasma membranes [154] and vesicular stomatitis virus [155]. In contrast to these systems, influenza A virus grown in Maden-Darby bovine kidney cells has been reported to have most of the sphingomyelin on the inner surface of the membrane [156]. In the case of viral membranes, it should be noted that most contain relatively high levels of sphingomyelin which reflect the similarity of lipid composition of the viral membrane to that of the plasma membrane of the host cell from which the membrane of the virus is derived [138,157,158].

VB. Changes in sphingomyelin distribution associated with aging and pathological conditions

During aging of the aorta and arteries in humans, there is a striking increase in the mole fractions of sphingomyelin and cholesterol in the membranes of cells comprising

these tissues. A similar, more pronounced increase occurs during the development of atherosclerosis [159,160]. Smith and Cantab [159] have shown that the principal change is in the intima where sphingomyelin reaches 40% of the total lipid during aging, and in advanced aortic lesions is as high as 70–80% of the total phospholipids. A striking increase in the proportion of sphingomyelin in the phospholipids of the intima with increasing severity of atherosclerosis has been reported in the fibrous plaques of the intima where the ratio of sphingomyelin-to-phosphatidylcholine increases 3- to 9-fold. This change is mainly in the 'amorphous' lipid fraction. A parallel increase in the cholesterol-to-phospholipid ratio has also been observed in this fraction [161]. These observations have been confirmed by Bottcher and van Gent [177] in studies on aorta and coronary arteries. They also noted that the ratio between saturated and unsaturated fatty acids increases with age. Work by Smith and Cantab [159] indicates that these changes in lipid composition cannot be explained by the changes in connective tissue.

Apparently, the increased concentration of lipids in the intima, of which 70% can be attributed to an increase in sphingomyelin, can be explained by changes in enzymatic activities [161,162], and by pronounced increase in the entry rate of the serum sphingomyelin into the aortic wall [161,163]. Eisenberg and coworkers [160] have described an increased incorporation of choline in the phospholipids of the aorta with increasing age of the animal. This increase parallels an increase of phospholipase A activity, however, sphingomyelinase activity remains constant or declines somewhat [160]. Similar results have been reported for normal human aorta by the same workers [162] who found that the sphingomyelin-to-phosphatidylcholine ratio changes linearly from a value of 0.4 at birth to a value of 2.4 at age 90. Concomitantly, the activity of the first enzyme in the sphingomyelin-degrading pathway declines from the level at birth to half that level at age 90. Enzyme activity levels in these studies were normalized using DNA as a reference. The authors suggest that this increase in phospholipids, especially sphingomyelin, is the result of either an increase in synthesis in the tissue or accumulation from plasma lipoproteins followed by an increased rate of hydrolysis of phosphatidylcholine, but not sphingomyelin.

The role of the serum lipoproteins as a source for aortic wall sphingomyelin is well demonstrated by Seth and Newman [163]. These authors show in rabbits that the level of sphingomyelin in the aortic intima increases exponentially with the time spent feeding on a cholesterol-rich diet. This increase is derived from the exponential increase in the entry rate of serum sphingomyelin into the aortic wall. Incorporation of [^{32}P]phosphate into sphingomyelin and other phospholipids in the perfused rabbit aorta does not increase with atheromatosis. The net result is an accumulation of sphingomyelin and a marked change in the ratio of sphingomyelin-to-phosphatidylcholine. Portman and coworkers [164,165] were able to show that most of the sphingomyelin accumulates in the agranular endoplasmic reticulum and in the plasmalemma. Smooth muscle cells, the principal cells of the intima and the inner media of the aorta wall, show these changes in lipid composition [164–166].

There is a significant positive correlation between the total cholesterol and the total amount of phospholipid in the aorta wall. The correlation between sphingomyelin and free cholesterol is more pronounced in the non-sudanophilic portion. The relation between endothelial integrity and accumulation of cholesterol during atherogenesis might be affected by the ability of the membranes of the endothelial cells to act as a barrier against excessive influx of cholesterol [167]. Bierman and coworkers [168] have shown that rat aortic smooth muscle cells take up 'remnant' of very low-density lipoprotein (VLDL)

formed by lipolysis of the VLDL by lipoprotein lipase. The remnant is cholesterol and sphingomyelin-rich [169,170]. Cultured human arterial cells preferentially bind and take up the low-density lipoprotein (LDL) and the VLDL fraction which includes the remnant [171]. LDL and the remnant are both rich in sphingomyelin and might be the source of the increase in the sphingomyelin level of the aortic wall [163].

Changes in lipid composition with age occur also in the nervous system. In humans, the rate of formation of new nerve membranes, and therefore the amount of total lipid in this tissue, is greater than the rate of loss by cell death into the fourth decade of life. During this period the rate of loss begins to exceed the rate of new membrane formation [52]. Throughout the lifetime of an individual, however, the lipid composition continually changes [52]. Rouser and coworkers [52] have shown that in human brain sphingomyelin and cerebroside gradually replace phosphatidylcholine and sulfatide replaces phosphatidylethanolamine. A similar phenomenon has been observed in other animal species. It is interesting to note, however, that in some invertebrates sphingomyelin is replaced by ceramide phosphorylethanolamine or ceramide phosphorylethylamine [52]. A similar age-dependent change in the ratio has also been noted for the lens of the eye in many species [172,173]. For humans, this change is most dramatic with the sphingomyelin content of the lens rising to as high as 70% of the total phospholipid and the phosphatidylcholine content falling to as low as 5% [172,174,175].

There are several overt pathological conditions in addition to atherosclerosis which are associated with large changes in tissue sphingomyelin content. The best known of these is Nieman-Pick disease in which a deficiency of lysosomal sphingomyelinase results in a marked decrease in the degradation rate of sphingomyelin [9]. The resulting accumulation of this phospholipid occurs in most cells and tissues of the body. The accumulation is usually paralleled by a marked increase in body cholesterol. The excess sphingomyelin is found concentrated primarily in lysosomes and in particular in the lysosomes of the large foam cells which are formed as a result of this disease [9]. The source of the accumulated sphingomyelin is of course, the result of the normal turnover of cellular membranes.

Changes in the sphingomyelin-to-phosphatidylcholine ratio have also been noted in muscular dystrophy [176] and in malignant disease. Leukemic cells appear to be deficient in both sphingomyelin and cholesterol [173,178]. Bergelson and coworkers [179] have found, however, that the sphingomyelin-to-phosphatidylcholine ratio increases 2.3-fold in Jensen hepatomas when compared to regenerating rat liver. Similar results have been obtained for other malignant systems [180–182]. In a variety of hepatomas the principal increase in sphingomyelin is seen in the mitochondrial and nuclear membranes [179,183], which normally have the lowest content of this phospholipid. In at least one type of hepatoma there is a marked increase in sphingomyelin. It is interesting to note that the level of sphingomyelin exchange protein is markedly elevated also in this hepatoma [184].

VC. Sphingomyelin and membrane integrity

Hydrolysis of the lipid components of biological membranes by suitable enzymes might be expected to result in lysis. It is surprising that hydrolysis of 80% of the sphingomyelin of the human erythrocytes to ceramide and phosphorylcholine by the sphingomyelinase of *Staphylococcus aureus* does not cause hemolysis [147]. In ruminant erythrocytes, which contain considerably more sphingomyelin than do human cells, extensive

action of the sphingomyelinase also fails to produce hemolysis [142,185]. Thus, under iso-osmotic conditions, the conversion of sphingomyelin to ceramide, which remains in the membrane, does not lead to loss of membrane integrity. Erythrocytes treated in this fashion are, however, osmotically fragile [147,156]. The conversion of erythrocyte sphingomyelin to ceramide also makes the membrane phosphatidylcholine available to attack by phospholipase C from *Bacillus cereus* with resulting hemolysis [147]. Thus, hemolysis is caused by exposure of the erythrocyte to both sphingomyelinase and *B. cereus* phospholipase C or to the nonspecific phospholipase C from *Clostridium welchii* which utilizes both sphingomyelin and phosphatidylcholine as substrates [147]. A similar situation is obtained for porcine erythrocytes [185] and for chicken erythrocytes depleted of ATP [145]. However, in ruminant erythrocytes which contain large amounts of sphingomyelin, although hydrolysis of sphingomyelin to ceramide is required for phospholipase C action on phosphatidylcholine, no hemolysis results. In contrast, in toad erythrocytes which contain small amounts of sphingomyelin, phospholipase C is able to promote hydrolysis of glycerophospholipids without prior hydrolysis by sphingomyelinase [145]. This hydrolysis leads to lysis if the cells are depleted of ATP [145].

The mechanical properties of erythrocyte membranes appear to correlate with sphingomyelin content. Cooper and coworkers [186] have shown that membranes obtained from acanthocytic erythrocytes in patients with abeta-lipoproteinemia are enriched in sphingomyelin and depleted in phosphatidylcholine. The sphingomyelin-to-phosphatidylcholine ratio can be as high as 1.56 in acanthocytes while the value in normal cells is about 0.86. Associated with this change is an increase in apparent microviscosity of the membrane as determined from 1,6-diphenylhexa-1,3,5-triene polarization studies. The acanthocytes also have a prolonged filtration time in a 0.3 μm nucleopore filter indicative of decreased membrane deformability [186].

A general positive correlation between membrane sphingomyelin content and apparent microviscosity has been shown for erythrocytes from a variety of mammalian species [103,187]. The stability of erythrocytes under iso-osmotic conditions is also higher for cells richer in sphingomyelin, although the resistance to osmotic shock appears to be lower [188]. Very similar relationships have been observed between the content of sphingomyelin mixed phosphatidylcholine/sphingomyelin multilamellar liposomes and apparent microviscosity and osmotic fragility [36].

The permeability of erythrocyte membranes from a variety of mammalian species to various nonelectrocytes correlates with the sphingomyelin content. The mole fraction of cholesterol is closely similar in the erythrocyte membranes of these species [188]. Deuticke [188] has also shown that changes in fatty acid composition have only a small effect on permeability. In general, the permeability to molecules for which no specific transport system exists decreases with increasing sphingomyelin content [188]. A strikingly similar correlation between sphingomyelin content and the permeability in multilamellar liposomes has been reported by Hertz and Barenholz [36]. Kirk [189] has shown that the level of active transport of K^+ in various mammalian erythrocytes is inversely related to the sphingomyelin-to-phosphatidylcholine ratio. Thus, the presence of sphingomyelin in the bilayer of the erythrocyte membrane is reflected in both the mechanical and permeability properties of the system. It is well to remember, however, that the erythrocyte membrane has the sphingomyelin located predominantly in the external surface unlike simple liposomal bilayers which probably have a symmetric distribution of this component [144].

VI. Summary

Sphingomyelin is one of the major lipids of the plasma membranes of mammalian cells. Together with phosphatidylcholine, the other choline-containing phospholipid, it makes up more than 50% of the total phospholipid in these membranes. In the plasma membranes of many cell types and over the course of diseases which affect cell membranes, although the total amount of these two lipids is constant, the membrane content of each of these phospholipids may vary greatly. Thus, it appears that these two choline-containing lipids are in certain measure interchangeable as membrane lipid components. This cannot, however, be the case because many of the physical characteristics of these molecules in bilayer systems are markedly different. Thus, variations in the relative amounts of sphingomyelin and phosphatidylcholine in bilayers and in biological membranes have profound effects on the system properties of the bilayer.

Perhaps the most striking difference between phosphatidylcholines and sphingomyelins derived from biological membranes are the temperatures of the gel-liquid crystalline phase transition exhibited by both of these types of molecules in bilayers. Most sphingomyelins have their transition temperatures in the physiological temperature range, while almost all naturally occurring phosphatidylcholines are well above their transition temperature at 37°C. Thus, mixed phosphatidylcholine/sphingomyelin bilayers containing more than 50 mol% sphingomyelin exhibit a transition near 37°C, while those containing less than this amount show no transition in this temperature range. This characteristic is also reflected in the apparent microviscosity of the mixed bilayer at 37°C which increases with increasing content of sphingomyelin. The phase behavior of bilayers comprised of these two choline-containing lipids is strongly influenced by the addition of cholesterol. There is compelling evidence to suggest that the interaction between sphingomyelin and cholesterol is much stronger than it is between phosphatidylcholine and cholesterol. Thus, the microscopic phase configuration of simple bilayer systems is markedly affected by the relative concentration of sphingomyelin, phosphatidylcholine and cholesterol. By inference, the same situation exists in the bilayers of the plasma membranes of cells.

The markedly different behavior of sphingomyelins and phosphatidylcholines in bilayer systems must reflect the differences in the molecular structures of these two classes of molecules. Although both molecular species have a polar region comprised of phosphorylcholine and a hydrophobic region comprised of two methylene chains, there are marked dissimilarities of structure elsewhere in the molecules. Phosphatidylcholines have two methylene chains of about equal length, while sphingomyelins have one methylene chain contributed by sphingosine which is of constant length. The other, contributed by the *N*-acyl group, is variable in length and can be up to 10 carbons longer than the sphingosine chain. This methylene chain length disparity in sphingomyelin is quite probably the basis, in part, for several interesting properties which are unique to bilayers composed of sphingomyelin. The generally lower degree of unsaturation of sphingomyelins relative to phosphatidylcholines also contributes to these differences. A third contributing factor is the difference in hydrogen bond-forming capability of the belt region which connects the polar and apolar regions of these molecules. The amide bond and hydroxyl group in this region of sphingomyelin can act as hydrogen bond donors while in phosphatidylcholine the carboxyl oxygens act as hydrogen bond acceptors. These differences in hydrogen bonding capabilities might be expected to be reflected in the interaction of these two lipids with other lipids in the bilayers and with membrane proteins.

It is clear that the properties of bilayers comprised of these two superficially similar

phospholipids reflect differences in molecular structure. Although the details of the relationships between molecular structure and properties and the system properties of bilayers comprised of these two phospholipid and cholesterols are not completely understood, much progress has been made. At the current level of this understanding, molecular explanations for certain of the physiologically important properties of biological membranes are beginning to emerge.

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