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Review

Biophysics of sphingolipids I. Membrane properties of sphingosine, ceramides and other simple sphingolipids

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Abstract

Some of the simplest sphingolipids, namely sphingosine, ceramide, some closely related molecules (eicosasphingosine, phytosphingosine), and their phosphorylated compounds (sphingosine-1-phosphate, ceramide-1-phosphate), are potent metabolic regulators. Each of these lipids modifies in marked and specific ways the physical properties of the cell membranes, in what can be the basis for some of their physiological actions. This paper reviews the mechanisms by which these sphingolipid signals, sphingosine and ceramide in particular, are able to modify the properties of cell membranes.
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Keywords: Sphingolipid; Ceramide; Sphingosine; Sphingosine-1-phosphate; Ceramide-1-phosphate; Membrane domain; Membrane order; Membrane permeability; Membrane fusion; Flip-flop

Contents

Abbreviations: DEPE, dielaidoyl phosphatidylethanolamine; DMPC, dimyristoyl phosphatidylcholine; DPH, 1,6-diphenylhexatriene; DPPC, dipalmitoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylserine; DSC, differential scanning calorimetry; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; SM, sphingomyelin

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1. Introduction

Sphingolipids have long been known as cell components. Their name was given by J.L.W. Thudichum in 1884, because of their enigmatic nature. In the second half of the past century, when the basic lipid metabolic pathways were unraveled, the catabolic and biosynthetic interrelations of sphingolipids became clear. In particular, ceramide and sphingosine were found to be intermediates in the metabolism of the more complex phospho- and glycosphingolipids [1,2]. More recently, with the discovery of the sphingolipid signalling pathway [3,4] the interest in these lipids was renewed. Very soon ceramide, and then sphingosine and other related compounds became established as lipid second messengers, or metabolic signals (see reviews in [5,7], and companion papers in this Special Issue).

At the time of the discovery of sphingolipid signalling very little was known of the properties of these lipids in membranes (for a review see [8]). Data for the simplest of these molecules, and also the most potent from the point of view of signalling, sphingosine and ceramide, were not available. The first detailed studies on the behavior of sphingosine in membranes were carried out by the groups of Gómez-Fernández and Kinnunen [9,10]. As for ceramide, Logfren and Pascher [11] and Maggio et al. [12] had performed some Langmuir balance measurements, and Pascher [13] had indicated in passing in an early paper the possibility that ceramides could perturb membrane permeability. Our own studies on ceramides started in 1996, precisely with the description of ceramide ability to permeabilize lipid bilayers [14].

At present there is a significant collection of data to allow a detailed description of the membrane properties of sphingosine and ceramides, and novel data on related sphingolipids are expected to appear soon. The present paper reviews the most relevant data in this lively area.

2. Chemistry of sphingosine and ceramides

2.1. Structures

The basic building block of sphingolipids is sphingosine, (2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol in current systematic nomenclature. According to an older, but extensively used, notation, its configuration is D-erythro-trans (Fig. 1). Other sphingosine-related molecules are also found in sphingolipids instead of the parent compound, for example the saturated dihydrosphingosine, or the saturated phytosphingosine (containing a third OH group in C4). The C1-phosphate ester of sphingosine is also an important molecule, commonly denoted as sphingosine-1-phosphate (Fig. 1).

Ceramides are N-acylsphingosines, and their C-1 phosphorylated forms, the ceramide-1-phosphates, are also known to exist in cell membranes (Fig. 1). Ceramides have been found in nature with fatty acids containing from 2 to 28 carbon atoms, either saturated or monounsaturated, in some cases containing an OH group in either C′2 (α-hydroxy fatty acids) or in the terminal carbon atom (ω-hydroxy fatty acids). Occasionally, a second fatty acid can be esterified to an OH group of sphingosine or related base. Ceramides constitute the hydrophobic backbones of all the complex sphingolipids: sphingomyelin, cerebrosides, gangliosides, etc. whose properties are discussed in the companion papers of this review [15,16]. Free ceramides are only found in large amounts in the skin stratum corneum [17]. They exist in much smaller proportions in cell membranes, in which they occur primarily as intermediates in the metabolism of the more complex sphingolipids, and where they play an important role in cell signalling [5,7]. The reader interested in ceramides in the context of lipid metabolism may consult Kanfer [2], Futerman [18], Mathias et al. [19], and Kolter et al. [20]. For a review on ceramides in natural lipids the reader is referred to Wiegandt [1] and Hakomori [21].

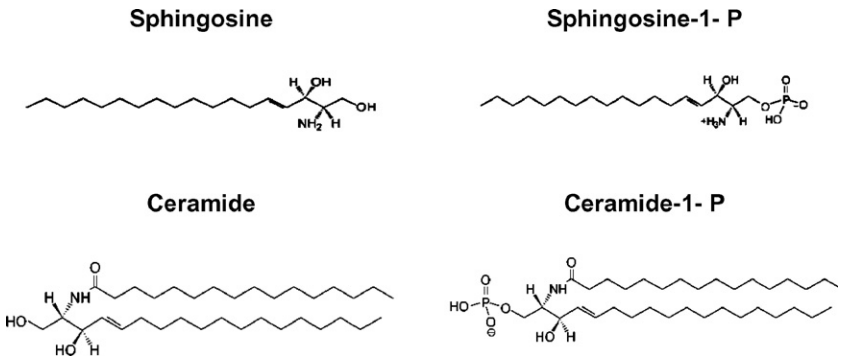

Fig. 1. Molecular structures of simple sphingolipids.

Dahlén and Pascher [22] determined by X-ray diffraction the crystal structure of N-tetracosanoylphytosphingosine, the first ceramide to be examined with that technique. In the triclinic crystal, the chains form an angle of 101°, so that the polar group is located in the apex of a V-shaped molecule. The molecules are linked together by one intra- and three intermolecular hydrogen bonds. As shown later by Pascher and Sundell [23], the presence of a dihydroxy fatty acid leads to a rather different overall appearance. N-(2D,3D-dihydroxyoctadecanoyl)-phytosphingosine molecules are almost fully extended in the unit cell, packing alternatingly with antiparallel orientation in single layers. The polar groups of the molecules, in this case including the two additional OH groups of the fatty acid, form an extensive network of hydrogen bonds. It should be noted, however, that both ceramides share, between them and with cerebrosides and phospholipids, a number of basic conformational features, and only differ with respect to a few torsion angles that are responsible for the overall apparent differences (Fig. 2) [24].

Li et al. [25] have established a number of interesting conformational features of ceramides by NMR spectroscopy. Ceramide was studied in diluted organic solutions that contained measured amounts of water. Their interpretation of the spectral trends points to a unique conformational arrangement adopted by the hydrophilic moiety of ceramide, regardless of acyl chain length. A network of cooperative H-bonds that involves the OH and NH groups of ceramide, as well as two water molecules, delineates a structural motif that is maintained even under conditions of complete hydration. The amide proton NH is donated to the OH groups on carbons C1 and C3 of the sphingosine backbone. The authors note, however, that some of these conformational features may differ from those adopted by ceramide in a bilayer arrangement.

Determining which of the structural features of sphingosine, ceramide, and their derivatives are essential for biological activity is still an ongoing task. In a comprehensive study of ceramide- and sphingosine-based inhibitors of the mitochondrial enzyme ceramidase, that cleaves the ceramide amide bond, Usta et al. [26] found that, for enzyme inhibition, the sphingosine analogue requires the C1 and C3 hydroxyl groups, the C4–C5 double bond in the trans configuration, and the NH protons. Dihydroceramides, that do not contain the double bond, are biologically inactive [27]. A synthetic L-threo-dihydrosphingosine, with potential anticancer use, is not desaturated in the cell, but is used instead as a precursor for the corresponding dihydrosphingomyelin [28]. The sphingosine double bond influences significantly the physical behavior of ceramides in monomolecular films at the air–water interface [29], and increases intramolecular hydration/hydrogen bonding in the ceramide polar region [25]. Interestingly, the cis configuration of the physiologically trans C4–C5 double bond modifies some, but not all of the cell functions of ceramide (G. Fabrías and F.X. Contreras, unpublished results). The length of the fatty acyl chain modifies significantly the physical properties of the corresponding ceramides [30–32], however in most cases short- and long-N-acyl chain ceramides display the same physiological properties [19,33,34]. Note however that, in a few instances, long- and short-chain ceramides have been found to have different biological effects [35,36].

2.2. Synthesis and determination

Organic synthesis of sphingosines is usually carried out from the "chiral pool". Thus, carbohydrates [37,38] and amino acids, specially serine-derived amino aldehydes and esters [39] have been extensively used. Other approaches include the use of chiral catalysts [40], chemoenzymatic approaches [41], as well as a variety of methods based on relayed asymmetric induction [42]. Milne et al. [43] have published a procedure in which the key step is a 1,2-metallate rearrangement of a higher order cuprate derived from an alpha-lithiated xylal derivative and tridecyllithium. Chun et al. [44] have described two methods for the preparation of ceramide analogues having the C4–C5 bond of the long-chain base as part of an aromatic or heteroaromatic system.

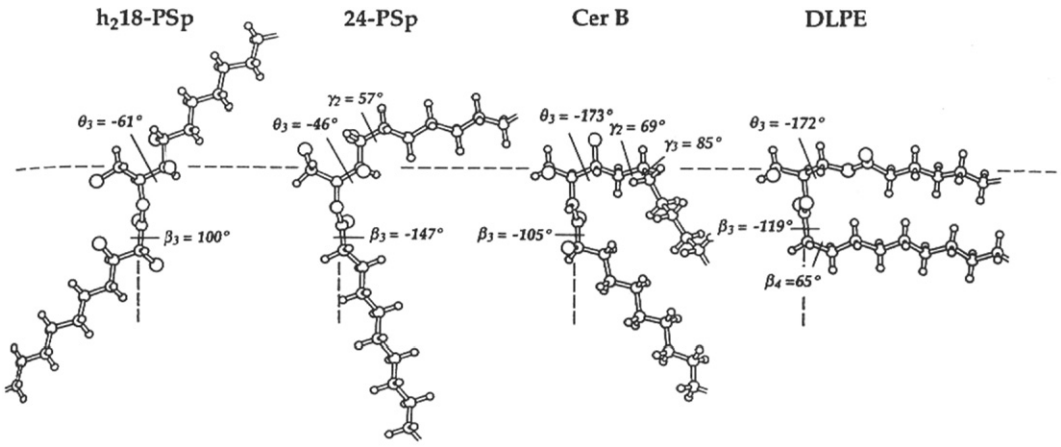

Fig. 2. Comparison of the chain conformation of a ceramide containing a hydroxylated fatty acid [h218-PSp, N-(2D, 3D-dihydroxyoctadecanoyl)-phytosphingosine], a "non-hydroxy" ceramide (24-PSp, N-tetracosanoylphytosphingosine), a cerebroside (Cer B, 1-β-D-galactosylceramide), and a phospholipid (DLPE, dilauroylphosphatidylethanolamine). In the two latter lipids the head group has been left out. The four molecules are arranged with the C(1)–C(2)–C(3) sphingosine/glycerol segment horizontally and the atoms C(1), C(3) and N [or O(2)] in the paper plane, in order to show the structural similarities of these molecules in the C(1)–C(2)–C(3) segment. Taken from Pascher and Sundell [23].

Quantitation of cellular sphingosine and ceramide levels has been achieved using thin-layer chromatography, high-performance liquid chromatography, or mass spectrometry (see [45], for a review). *Escherichia coli* diacylglycerol kinase has also been applied in this respect, although diacylglycerol obviously interferes. Recombinant human ceramide kinase provides instead a highly specific enzymatic method for quantifying long-chain ceramides in cellular lipid extracts [46].

3. Physical properties of sphingosine

Sphingosine was first found to have a biological effect by Hannun et al. [47], who described protein kinase C inhibition in human platelets caused by the simplest of sphingolipids. The molecular parameters (molecular area, surface potential, collapse pressure, dipole moment contribution) of sphingosine in monolayers at the air/water interface were measured by Perillo et al. [48]. Sphingosine behaves as a surface-active amphiphile, with a critical micellar concentration in the 20 μM range [49]. In the fully protonated form, at pH 6.0, pure sphingosine exhibits a thermotropic transition centred at 30 °C. This transition is shifted to 39 °C at pH 10 [9]. Also López-García et al. [9,50] observed, using ^{31}P-NMR, that sphingosine in membranes had an apparent pK_a=9.1, thus it is positively charged under physiological conditions. Because of this, sphingosine may interact differently with anionic and zwitterionic phospholipids in membranes. In mixtures with the anionic dipalmitoyl phosphatidylserine DPPS, sphingosine was seen to rigidify the membranes, forming an azeotropic mixture at a DPPS/sphingosine molar ratio of 2:1. A eutectic point was observed at 85 mol% sphingosine in DPPS. ^{31}P-NMR showed the presence of a lamellar phase at DPPS/sphingosine molar ratios lower than 1:1, whereas at higher molar ratios an isotropic component was detected [9].

In mixtures with zwitterionic phospholipids, sphingosine behavior was also different with the "zero curvature" lipid dipalmitoyl phosphatidylcholine (DPPC) and with the "negative curvature" inducer dielaidoyl phosphatidylethanolamine (DEPE). With DPPC sphingosine increased the so-called pre-transition temperature (T_p, the temperature at which the gel-to-rippled phase transition occurs in certain saturated phospholipids) up to 10 mol%. Higher sphingosine concentrations abolished the pre-transition [10]. Otherwise the interaction of sphingosine with DPPC was rather similar than with DPPS, sphingosine was equally found to increase the gel–fluid transition temperature, T_m, i.e. to rigidify the membranes, with an azeotropic point at 2.3:1 DPPC/sphingosine molar ratio, and a eutectic point at 90 mol% sphingosine. At variance with DPPS, however, sphingosine in DPPC gave rise to lamellar phases, according to ^{31}P-NMR, in all the range of concentrations [50]. With DEPE, sphingosine slightly lowered the T_m main transition temperature, i.e. fluidized the bilayer, forming no azeotropic point. A eutectic point was observed at 60 mol% sphingosine in DEPE, with solid-phase immiscibilities being present between 50 and 85 mol% sphingosine. Unlike DPPS and DPPC, DEPE displays a lamellar-to-hexagonal phase transition in an easily accessible temperature range, ca. 65 °C. Sphingosine, at concentrations below 33 mol%, slightly shifted the transition to lower temperatures, but the transition disappeared abruptly at higher sphingosine proportions, so that isotropic phases were formed instead [50].

In contrast with DPPC and other diacylphosphatidylcholines, the T_p pre-transition temperature for dihexadecyl phosphatidylcholine was lowered by sphingosine, showing that the sphingolipid destabilizes the interdigitated gel phase typical of dialkylphospholipids [10]. In mixtures with egg phosphatidylcholine, a lipid whose T_m is below 0 °C, the presence of equimolar sphingosine shifted the main transition to 29 °C. Measurements of surface pressures in lipid monolayers at the air–water interface revealed that sphingosine was able to "condense" egg phosphatidic acid monolayers, i.e. to reduce the average area occupied by one lipid molecule [10], in agreement with the observations of bilayer rigidification by sphingosine.

Gómez-Fernández and co-workers have studied the influence of sphingosine on the interaction of phosphatidylserine with Ca^{2+}. This interaction is important in a number of physiological processes [51]. Ca^{2+} abolished the gel–fluid phase transition of DPPS at DPPS/Ca^{2+} molar ratios of 1:0.5 or lower. However, DPPS/sphingosine/Ca^{2+} mixtures exhibited detectable phase transitions even at 1:1:10 mole ratios. In fact, the transition enthalpies were essentially the same for DPPS/sphingosine equimolar mixtures in the presence and absence of Ca^{2+}. Calcium binding experiments using $^{45}Ca^{2+}$ demonstrated that sphingosine all but abolishes Ca^{2+} binding by DPPS at equimolar ratios [51]. The system was further studied using IR spectroscopy, examining Ca^{2+}-induced dehydration due to the very strong electrostatic interaction established between DPPS and sphingosine. It was also found that the apparent pK_a of the DPPS carboxylate group decreased from 4.6 in pure DPPS to 2.1 for DPPS/sphingosine equimolar mixtures [52]. The ensemble of these results shows that sphingosine, being a positively-charged molecule under physiological conditions, prevents the binding of cations such as Ca^{2+} through electrostatic charge neutralization.

Because of its cationic nature at neutral pH, sphingosine has been used in the preparation of positively charged liposomes for the study of membrane binding to a variety of macromolecules, including DNA and enzymes. In liposomes composed of egg phosphatidylcholine and egg phosphatidic acid (85:15, mol ratio), the mainly electrostatically controlled binding of adriamycin and cytochrome *c* was reversed upon the inclusion of increasing contents of sphingosine [53]. Comparable results were obtained with sphingosine/egg phosphatidic acid monolayer studies in which penetration of either cytochrome *c* or adriamycin was progressively reduced by increasing sphingosine contents in the monolayers. Mustonen et al. [53] also studied the effects of sphingosine on phospholipase A_2 activity. Under low Ca^{2+} concentrations, that limit the rate of the enzymatic reaction, sphingosine gradually inhibited the hydrolysis of phosphatidylcholine and at 1:6 sphingosine/PC mol ratio a nearly complete lack of hydrolysis was evident. Franson et al. [54] described as well the inhibition of both phospholipase A_2 and phospholipase D by sphingosine. Finally, the influence of

the net positive charge of sphingosine on protein binding to bilayers has also been made apparent in the demonstration that this sphingolipid promotes binding of the assembly factor P17 from bacteriophage PRD1 to lipid bilayers [55].

Kinnunen et al. [56], using a combination of fluorescence spectroscopy, fluorescence microscopy, and differential scanning calorimetry observed that binding of DNA to a bilayer composed primarily of egg phosphatidylcholine depended critically on the presence of sphingosine in the liposomes. Attachment of DNA to sphingosine-containing membranes could be reversed by the further inclusion of the negatively-charged phosphatidic acid in the bilayers. On the basis of these studies, Paukku et al. [57] prepared sphingosine-containing liposomes for the *in vitro* transfection of DNA. KK-1 murine granulosa cells were most efficiently transfected with liposomes composed of phytosphingosine/dioleoyl phosphatidylethanolamine/dioctanoylglycerol (64:31:4.8 mole ratio). Other cell lines could also be effectively transfected with these liposomes, but the murine Sertoli cells, MSC-1, selectively resisted transfection by the sphingosine-containing liposomes.

The interaction of DNA with sphingosine/phosphatidylcholine mixtures has been studied in more detail also by the Kinnunen group, using the Langmuir balance. Lipid monolayers were spread consisting of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and sphingosine. Surface pressure-area isotherms and monolayer dipole potentials were determined as a function of sphingosine mole fraction with and without a charge saturating concentration of DNA in the subphase. In agreement with previous observations [10], Saily et al. [58] found that even low contents (5 mol%) of sphingosine in a POPC monolayer caused remarkable film condensation (by 20% at 20 mN/m). This was accompanied by a pronounced increase in dipole potential, suggesting that condensation causes a reorientation of the $P^{-}N^{+}$ dipole of the POPC headgroup. The presence of DNA affected the POPC/sphingosine monolayers differently, depending on the proportion of sphingosine.

A novel, interesting property of sphingosine is its ability to permeabilize membranes to small solutes. This was recently shown by Siskind et al. [59] who, using electrophysiological methods, described sphingosine "channels", characterized by short open lifetimes and diameters smaller than 2 nm, in planar lipid bilayers. Sphingosine was also found to form these channels in red blood cell membranes. This sphingosine property has also been explored by Contreras et al. [49], who found sphingosine-induced efflux of aqueous solutes from liposomes and resealed ghost membranes (Fig. 3). Sphingosine increased permeability in a dose-dependent form, between 5 and 20 mol% total lipids. The authors interpreted this effect as a result of the bilayer rigidifying properties of sphingosine, mentioned above. Rigidification would stabilize gel domains in membranes, raising their melting temperatures and increasing the transition cooperativity. Structural defects would form as a result of the lateral phase separation of the "more rigid" and "less rigid" domains, and these defects would be likely sites for the leakage of aqueous solutes to the extravesicular medium.

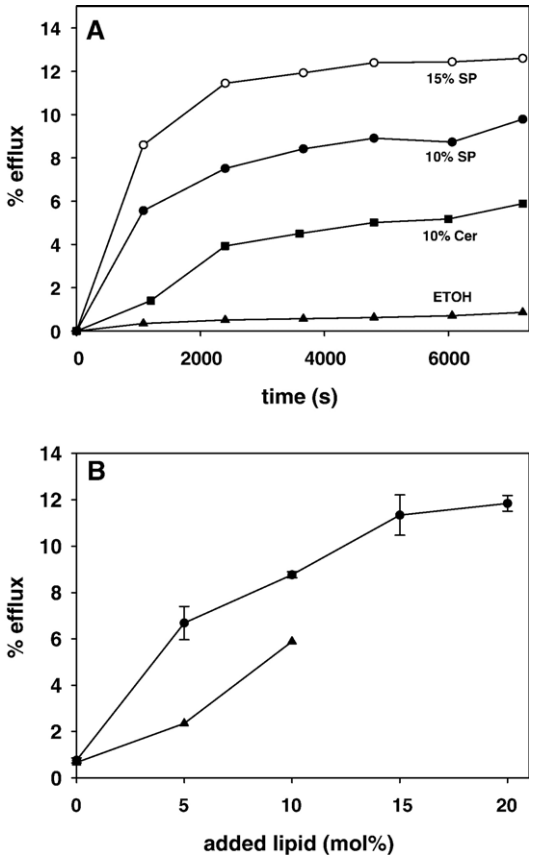

Fig. 3. Sphingosine- and ceramide-induced efflux of vesicular aqueous contents. LUVs were prepared, composed of SM/PE/Ch (2:1:1, mol ratio) containing entrapped ANTS/DPX. Sphingosine or ceramide in ethanol were added at time 0. (A) Time course of efflux. (▲) Ethanol (control); (■) +10 mol% ceramide; (●) +10 mol% sphingosine; and (○) +15 mol% sphingosine. (B) Dose–response curves. Data at time=6000 s. (▲) Ceramide; (●) sphingosine (average values± SD, n=3). Taken from Contreras et al. [49].

The presence of coexisting domains in the plasma membrane makes it a target for sphingosine permeabilization.

3.1. Sphingosine-1-phosphate

This bioactive lipid was found to serve as a second messenger for cell proliferation [60], and survival [61]. More details on its physiological actions can be found in a companion paper in this Special Issue [62]. Very little is known of the biophysical properties of this lipid. Work in progress in our laboratory indicates that pure sphingosine-1-phosphate in buffer exhibits a low-enthalpy thermotropic order-disorder transition centered at 65.2 °C. In common with sphingosine it lowers the temperature of the gel–fluid transition of DEPE, thus stabilizing the fluid over the gel phase, but at variance with the non-phosphorylated sphingolipid it clearly hinders the lamellar–hexagonal transition, stabilizing the bilayer over the inverted hexagonal structure. This would be expected, in view of the opposite geometries of DEPE and sphingosine-1-phosphate, the former favouring negative monolayer curvature, and inverted hexagonal phases, the latter inducing positive curvature, and micelle formation.

4. Ceramides

Ceramides (N-acylsphingosines) have been known for decades as intermediates in sphingolipid metabolism and as minor membrane components [1,63]. However, their role as powerful metabolic signals involved in many cellular events has been discovered more recently [3,4]. The most commonly found ceramides, that is, those with a fatty acyl chain of 16 carbon atoms or longer, are among the least polar, more hydrophobic, lipids in membranes. Indeed, their hydrophobicity explains their abundance in the stratum corneum, the barrier that prevents water evaporation through the skin [64]. Their solubility in water is negligible. Thus, free ceramides cannot exist in solution in biological fluids or in cytosol. In addition, long-chain ceramides (e.g., with a fatty acid C12 or longer) belong to the category of "non-swelling amphiphiles" [65], implying that they cannot even give rise to micelles or other aggregates in aqueous suspension, as do, for example, common phospholipids, glycosphingolipids, surfactants, etc. Short-chain ceramides (e.g., N-acetyl or C2 ceramide) appear to "swell" in water, giving rise to homogeneous dispersions [66,67], that are treated, in practice, as solutions.

4.1. Properties of the pure ceramides

The thermotropic properties and phase behavior of anhydrous and hydrated pure ceramides have been examined in detail by Shipley and co-workers. A synthetic N-palmitoylsphingosine (C16 ceramide) was studied at various degrees of hydration [68], using differential scanning calorimetry and X-ray diffraction techniques. The fully hydrated ceramide exhibits a complex polymorphic behavior. At room temperature, a well-ordered metastable bilayer phase exists. Upon increasing the temperature, an exothermic transition occurs at 64.2 °C to form a stable bilayer phase with crystalline chain packing. The transition produces an increased chain tilting with respect to the bilayer normal and/or partial dehydration. Further heating converts the crystalline phase into a disordered, melted-chain phase, of an undefined structure, through an endothermic transition at 90 °C (ΔH=13.8 kcal/mol). In contrast, the anhydrous C16 ceramide shows a single, broad endothermic transition at 95.4 °C (ΔH=10.4 kcal/mol). The complex behavior of the hydrated ceramide is also found in more polar sphingolipids, for example sphingomyelin or cerebroside, and suggests hydrogen bonding of the hydroxyl groups of sphingosine to the amide group of adjacent molecules. However the presence of an α-hydroxy group in the fatty acyl residues induces a simpler phase behavior, and the fully hydrated hydroxy ceramides exhibit a single, reversible endothermic transition between a bilayer gel and a hexagonal fluid phase [69]. Similar results have been independently obtained by Han et al. [70].

Phase behavior as well as intermolecular and intramolecular interactions in hydrated ceramides have been examined by Moore et al. [70 bis] using infrared spectroscopy. Ceramides of natural origin have been studied, either containing or not α fatty acids. The observed phase behavior is mostly coincident with the one described by Shah et al. [68,69], the differences being probably due to the complex fatty acid composition of the ceramides used by Moore et al. In addition, infrared spectroscopy allows the observation of extensive hydrogen-bonding between ceramide headgroups in the bilayer phase. Importantly, intermolecular interactions in non-hydroxy ceramides occur mainly along an axis perpendicular to the bilayer plane, while hydroxy ceramides exhibit in-plane interactions through hydrogen-bonding. This behavior may help to explain the phenomenon of lateral segregation of non-hydroxy ceramides when mixed with phospholipids (see Domain formation).

Langmuir balance studies have been very useful in understanding certain ceramide properties. In principle, a lipid monolayer at the air–water interface can be obtained either by carefully depositing the lipid (in organic solution) on top of the aqueous surface, or else by injecting the lipid solution into the aqueous phase. In both cases the equilibrium situation will include a lipid monolayer at the interface. In practice, however, when a long-chain (N-palmitoyl) ceramide in organic solution is injected in the aqueous phase (the "subphase") surface tension does not vary in several hours, such is the hydrophobicity of the ceramide, that remains presumably in the form of aggregates in the trough [31]. However when the same ceramide is deposited onto the water surface, a monolayer is formed, as indicated by the decrease in surface tension detected by the balance. The extreme hydrophobicity of ceramides, demonstrated in these experiments, explains the very low rate of exchange of long-chain ceramides between phospholipid vesicles (in the order of days) [71] and the need for a ceramide transfer protein in cells [71 bis].

Studies of bovine brain ceramides containing mainly 32% stearic and 48% nervonic acids, in monolayers extended at the air–water interface of a Langmuir balance, indicate that ceramide forms a liquid-condensed film at 24 °C with a limiting mean molecular area of 0.40–0.45 nm^2 and surface potential of 515 mV at the collapse pressure of 40 mN/m [8,12]. Similar mean molecular areas, with collapse pressures at ca. 50 mN/m have been measured for pure N-palmitoyl and N-nervonoyl ceramides [72]. The compression free energy (the two-dimensional work required to bring together the molecules from the liquid expanded state at 2 mN/m to a closely packed state at 35 mN/m) of bovine brain ceramides is of about 200 cal/mol [8], significantly lower than that of the more complex sphingolipids and phospholipids. Conversely the maximum compressibility modulus (a quantitative measure of the state of the monolayer, obtained from the slope of the pressure-area curve as $K=-A\,(\delta\pi/\delta A)_T$, A being the molecular area at a given surface pressure) of bovine brain ceramide has been established at 205 [73], higher than those found for the more complex membrane lipids, and indicating a rather condensed state of the ceramide monolayer. Even higher maximum values of K (or Cs^{-1} in the terminology of Brockman and co-workers) are found for chemically defined ceramides, ca. 400 for N-nervonoylsphingosine and ca. 700 for N-palmitoylsphingosine [72]. The higher the value of this modulus, the lower the interfacial elasticity [74].

5. Ceramides in mixtures with phospholipids

When mixed with phospholipid monolayers or bilayers, ceramides have two main effects, they increase the molecular order of phospholipids, an they give rise to lateral phase separation and domain formation. Ceramides in bilayers have the additional effects of inducing membrane permeabilization, transbilayer (flip-flop) lipid movements, and transition to non-lamellar phases. The effects of ceramides in mixtures with the more complex glycosphingolipids are described in the companion review by Maggio et al. [16].

5.1. Lipid ordering effect

The degree of lipid chain order (in particular the "dynamic order" that is usually related to bilayer fluidity) is often estimated using the fluorescence probe diphenylhexatriene (DPH). DPH fluorescence polarization increases with increasing molecular order (decreasing fluidity). In fact, a natural ceramide (containing both α-hydroxy and non-hydroxy long-chain fatty acids) was seen to increase acyl chain order in a dose-dependent form in dimyristoyl phosphatidylcholine bilayers in the fluid lamellar phase [75]. A similar effect was observed with a chemically defined C16-ceramide in bilayers composed of 1-palmitoyl-2-oleoyl phosphatidylcholine [76]. Moreover, in situ generation of ceramide through the action of sphingomyelinase on bilayers containing phosphatidylcholine and sphingomyelin also lead to an increased DPH fluorescence polarization [76]. Massey [77] also observed an increase in DPH polarization as a function of ceramide concentration in sphingomyelin, POPC and DPPC bilayers in the fluid phase. Addition of ceramide to DPPC and sphingomyelin bilayers, both of which have gel–fluid phase transitions at ca. 40 °C, increased the phase transition temperature T_m, in agreement with the ordering effect of ceramide. Recently, Silva et al. [78] have shown that polarization of transparinaric acid fluorescence in POPC fluid bilayers is much more sensitive than DPH to the ceramide-induced ordering effect of ceramide, an increase being detectable already at 2 mol% ceramide, and reaching a plateau at 20 mol%. The mean fluorescence lifetime of transparinaric acid also increases sharply with ceramide concentration. The increased lifetimes would in principle lead to a decrease in anisotropy, but the rigidity imposed by the ceramide is such that the increased rotational correlation times compensate for the raised lifetimes [78].

A different technique, namely ^{2}H-NMR spectroscopy, was used by Huang et al. [79,80] to show that either bovine brain ceramide or synthetic C16-ceramide induced large increases in the order parameters of the acyl chains of fully deuterated dipalmitoylphosphatidylcholine (d_{62}-DPPC) when the latter is in the fluid state. More recently, Hsueh et al. [81] observed using ^{2}H-NMR that the acyl chains of d_{31}-POPC in the fluid phase were ordered by the presence of ceramide. They were also able to detect that, under those conditions, ceramide chain ordering was greater then that of POPC. The ordering effect of ceramides is in good agreement with the "very rigid" Langmuir-type monolayers that were formed by different long-chain natural and synthetic ceramides at the air–water interface [82], and with the highly condensed ceramide monolayers described by Carrer and Maggio [73]. Note however that, in mixtures with the fully saturated DPPC, ceramide mixes almost ideally, at least at 24 °C [73], thus no condensing effect on the phospholipid is exerted here. Consequently there may not be a simple correlation between lipid condensation in monolayers and lipid chain ordering in bilayers.

5.2. Domain formation

Separation into ceramide-rich domains was first observed by Huang et al. [79], who examined the structure of bilayers composed of fully deuterated DPPC and bovine brain ceramide using ^{2}H-NMR spectroscopy. These authors observed that addition of ceramide induced lateral phase separation of fluid phospholipid bilayers into regions of gel and liquid–crystalline (fluid) phases, ceramide partitioning largely into the gel phase of d_{62}-DPPC. Further ^{2}H-NMR studies by Hsueh et al. [81], of mixtures of C16:0 ceramide and d_{31}-POPC found that gel and liquid–crystalline (fluid) phases coexisted over a wide range of temperatures and compositions, with domains of different composition and physical state being present at physiological temperatures. However, no evidence of liquid–liquid phase separation in the fluid phase was found. A similar behavior of long-chain ceramides has been detected in phosphatidylcholine/phosphatidylserine mixtures [83]. The use of a pyrene-labeled phospholipid, a fluorescent probe that is sensitive to lateral mobility and to the local concentration of fluorophore in the membrane, allowed Holopainen et al. [75,76] to detect ceramide-enriched microdomains in fluid phosphatidylcholine membranes. A parallel kinetic study of ceramide generation and formation of ceramide-rich domains [76] revealed that, under conditions allowing fast ceramide synthesis, changes in the lateral organization of the membrane took significantly longer time to occur (one order of magnitude, under the conditions of the experiment).

Domain formation by ceramides was also described by a combination of differential scanning calorimetry and IR spectroscopy, using natural ceramides (brain, egg) and several synthetic phospholipids [84]. Calorimetry was used to detect gel–fluid transitions. Different domains, when formed, "melt" at different temperatures, so that they can be easily detected. Veiga et al. [84] found lateral separation of ceramide-rich domains with as little as 5 mol% ceramide. Disparity of chain lengths between lipid species ("chain mismatch") has been mentioned as the origin of certain cases of lateral phase separation [85]. However this is not the case in the present example, because ceramides of different lengths (brain, egg) gave almost equivalent results in phospholipid mixtures examined by calorimetry. IR spectroscopy, together with the use of deuterated lipids, can be applied to the resolution of the gel–fluid transition in ceramide-rich and -poor domains. Veiga et al. [84] prepared mixtures of deuterated phospholipid (d_{54}-DPPC) with natural ceramide. IR spectroscopy clearly reveals a gradual melting of DPPC along a wide range of temperatures (covering the various domains), while ceramide exhibits a much sharper transition,

corresponding to the high-temperature melting, ceramide-enriched domains [84].

Carrer and Maggio [73] have studied mixtures of bovine brain ceramide, which contains mostly C18:0 and C24:1 fatty acids, with DPPC, both by differential scanning calorimetry and in lipid monolayers extended at the air–water interface. The calorimetric results are essentially coincident with those by Veiga et al. [84], with in-plane phase separation being hinted at with only 1% ceramide. Measurements of surface potentials in monolayers reveal that molecular dipole potentials may play an important role in lateral phase separation, with favourable ceramide-PC dipolar matching in the liquid state being one of the local determinants for close molecular interactions, and unfavourable matching explaining domain segregation of ceramide-enriched phases (see also [71]). The extensive review by Maggio [8] contains detailed information on domain formation in sphingolipid-based membranes.

An important observation related to domain formation by ceramides in phospholipid bilayers comes from the studies by ten Grotenhuis et al. [82] involving ceramides, cholesterol, and free fatty acids, that is, the lipids believed to constitute the stratum corneum matrix. These authors examined an extensive series of binary and ternary mixtures of the above lipids in the form of supported Langmuir–Blodgett monolayers. The monolayers were probed by atomic force microscopy. C16-ceramide mixed well with cholesterol, but the longer-chain ceramides did not, and lateral phase separation was observed under a variety of conditions. The same phenomenon of in-plane separation was described by Ferraretto et al. [86] for sphingolipids other than ceramides, using differential scanning calorimetry. With palmitoylsphingomyelin bilayers containing bovine brain (long-chain) GM1 ganglioside and cholesterol, these authors were able to detect separate domains enriched in sphingomyelin and cholesterol. In a recent study Chiantia et al. [87] have used a combined approach of atomic force microscopy, fluorescence correlation spectroscopy, and confocal fluorescence imaging to observe gel-like ceramide-enriched domains in SM/DOPC/Cholesterol/ceramide supported bilayers in the liquid-ordered state.

Monolayer studies have revealed interesting aspects of domain formation by ceramide in phospholipid monomolecular films. Holopainen et al. [72] studied the mixing behavior of dimyristoyl phosphatidylcholine (DMPC) and either N-palmitoylsphingosine (C16: ceramide) or N-nervonoylsphingosine (C24:1 ceramide). C16:0 ceramide appears to be immiscible with DMPC, while C24:1 ceramide and DMPC are miscible, albeit non-ideally. In both cases fluorescence microscopy reveals the segregation of ceramide-rich and-poor domains, but with very different morphologies. For C16:0 ceramide the dark, ceramide-enriched domains seen under the microscope exhibit a complex, interconnected network with some round domains entrapped into the bright continuum. C24:1 ceramide/DMPC monolayers exhibit flower-like ceramide-enriched, solid domains that do not fuse together even at high surface pressures. Domain shape is the result of a balance between line tension and dipole–dipole repulsion. When the former dominates, round domains with minimum domain boundaries arise, while flower-shaped domains and networks are the result of predominant dipole–dipole repulsion [88]. The different domain morphologies found for the two ceramides in mixtures with DMPC are probably reflecting the different interaction between the film constituents. In spite of the fact that observations in lipid monolayers cannot be directly translated into phenomena occurring in membrane bilayers, the results by Holopainen et al. [72] suggest that different ceramide species may serve very different biological functions, determined by their different impact on the membrane physical properties.

A recent, detailed study of C16 ceramide/POPC interaction in fluid bilayers [78] sheds new light on the ability of ceramides to give rise to rigid, ordered domains under these conditions. These authors have combined fluorescence spectroscopy and transmission electron microscopy to construct a detailed phase diagram for the binary mixtures of the above lipids. The phase diagram is dominated by spherical vesicles in the fluid phase at low ceramide concentrations and high temperatures. Lowering temperature leads to coexisting fluid and ceramide-rich gel phases, and eventually to coexisting POPC-rich and ceramide-rich gel phases. At ceramide concentrations above 50 mol% a new highly ordered ceramide-rich phase appears. Also at these high ceramide concentrations cylindrical membranous structures with round extremities are visible under the electron microscope, while crystalline ceramide structures are only seen above 92 mol% ceramide. In agreement with several previous studies, Silva et al. [78] find significant changes in membrane properties already at 2 mol% ceramide. This, together with their observation that ceramide-rich domains are large in size, supports the biological relevance of these studies, considering that ceramide-driven formation of large liquid-protein "platforms" in cell membranes has been proposed to have a key role in viral and bacterial internalization, and in the induction of cell apoptosis by death receptors and stress stimuli [89–91].

In the apoptotic signalling pathway, ceramide is believed to be formed as a consequence of acid sphingomyelinase activation, in response to an appropriate stimulus (see reviews in [5,6,92]). Thus biophysical studies of domain formation in ceramide/sphingomyelin mixtures are particularly relevant in this context. As mentioned above, Holopainen et al. [76] observed formation of ceramide-rich domains treating sphingomyelin-containing vesicles with bacterial sphingomyelinase. Further studies form Maggio's laboratory have provided interesting data on the subject of ceramide domains generated by sphingomyelinase. Epifluorescence studies of sphingomyelin monolayers being degraded by sphingomyelinase added to the aqueous subphase, indicate in real time that formation of ceramide initially alters surface topography by inducing phase separation into condensed (ceramide-enriched) and expanded (sphingomyelin-enriched) domains [93] (Fig. 4). The ceramide-enriched phase grows steadily as the reaction proceeds at a constant rate, up to the "percolation point", when the condensed domains coalesce into a continuous one that contains the now discontinuous expanded phase. At this point the reaction rate drops rapidly, showing that the supramolecular organization into domains influences enzyme activity at the local molecular level. Also significant is the observation by Fanani et al. [93] that the

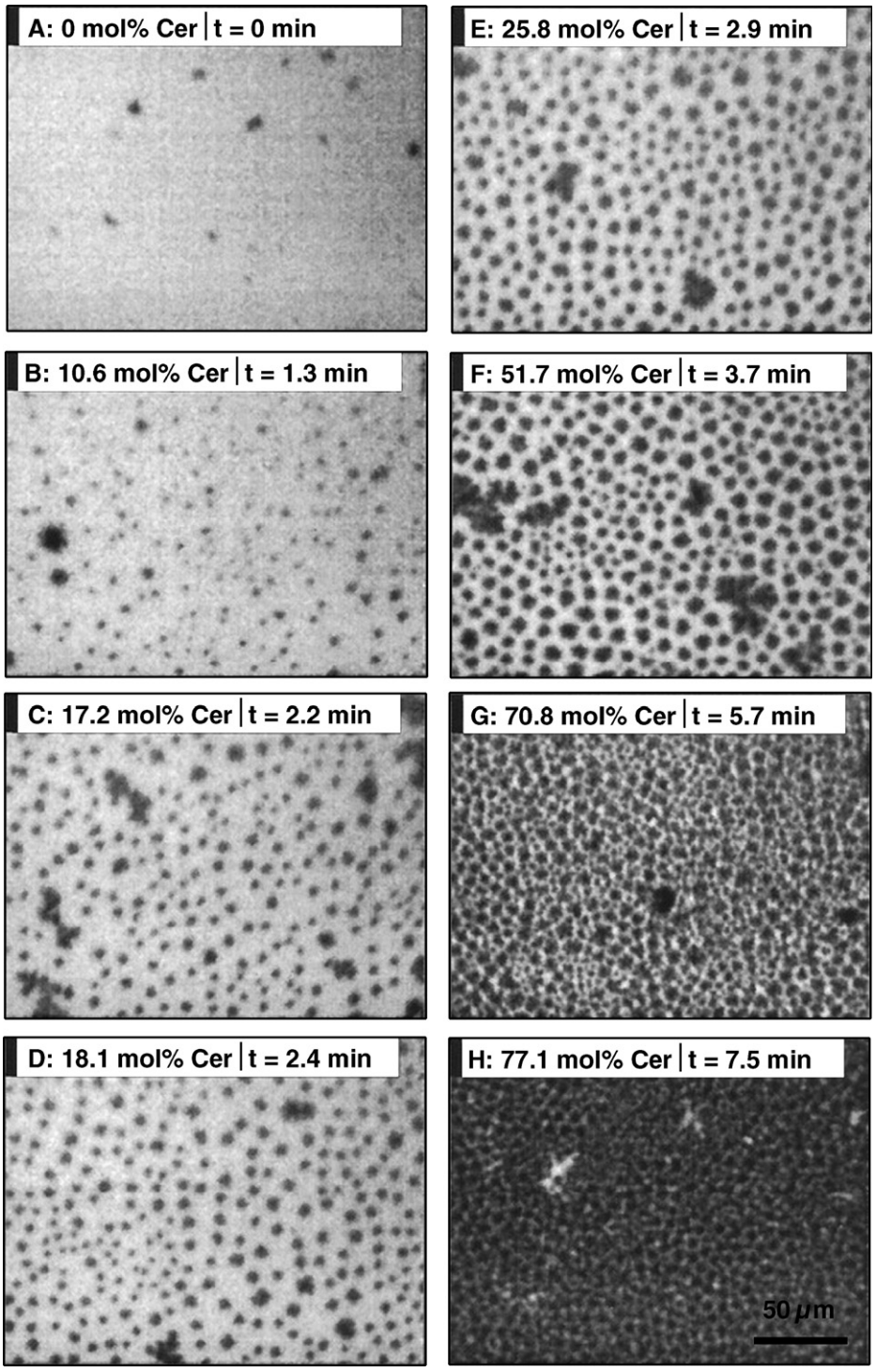

Fig. 4. Time course of sphingomyelinase-driven conversion of SM to ceramide in lipid monolayers at 10 mN/m. (A–H) Representative pictures of $DiLC_{12}$ fluorescence in SM/Cer monolayers at different times of the enzymatic reaction. Dark regions in the pictures represent the formation of ceramide-enriched lipid phases with unfavourable partition conditions for the lipophilic fluorescent probe $DiIC_{12}$. Taken form Fanani et al. [93].

surface topography derived from the sphingomyelinase-driven reaction is different from that obtained by premixing sphingomyelin and ceramide in the same proportion, indicating that the information contents depends on the manner in which the surface was generated.

A more detailed study of the same system, in which advanced image-processing routines were incorporated in combination with time-revolved epifluorescence microscopy [94] revealed the following sequence of events: (1) spontaneous nucleation and circular growth of ceramide-enriched domains after injection of sphingomyelinase into the aqueous subphase or the sphingomyelin monolayer; (2) domain-intrinsic discrete transitions from circular to periodically undulating shapes, followed by a second transition toward increasingly branched

morphologies; (3) lateral superstructure organization into predominantly hexagonal domain lattices; (4) formation of super-superstructures by the hexagonal lattices, and (5) rotationally and laterally coupled domain movement before domain border contact. Again none of these patterns could be reproduced when ceramide was added to sphingomyelin instead of being generated by the enzyme. Härtel et al. [94] conclude that sphingomyelinase induces a domain-specific packing and orientation of the molecular dipole moments perpendicular to the air/water interface. The observed high-level organization of lipid domains constitutes a novel example of signal transduction from local (nm, single molecule) to long-range (μm, domain superstructure) scales.

Ceramide-enriched domains in sphingomyelin/ceramide vesicle bilayers, rather than monolayers, have been described recently by Sot et al. [95], using mainly differential scanning calorimetry (DSC) and fluorescence microscopy. DSC data showed for pure egg sphingomyelin a rather narrow transition centered at 39 °C. Egg ceramide, even at low proportions (5 mol %) had the effect of widening the phase transition, shifting it to higher temperatures. More important, the endotherms of ceramide-containing samples had a clearly asymmetric shape, indicating formation of high-T melting ceramide/sphingomyelin domains. In fact the observed overall endotherms could be fitted to three-component endotherms, corresponding to as many coexisting domains with different compositions. The corresponding phase diagram includes a wide temperature/composition region of coexisting domains [95]. In addition, a series of observations were carried out on giant unilamellar vesicles (GUV) using fluorescence microscopy. The vesicles were doped with the fluorescent probe DiI_{C18}, that partitions preferentially into the more fluid membrane regions. Pure sphingomyelin vesicles appeared uniformly stained, whereas those containing egg ceramide displayed dark areas, corresponding to rigid, ceramide-rich domains. Increasing ceramide concentrations caused a parallel increase in dark areas, that changed their shape from circular to elongated or worm-like. The percolation point for the ceramide-rich domains appeared to occur between 20 and 30 mol% ceramide, at the latter concentration the dark domain being already continuous [95]. Interestingly, these authors found that sphingomyelin/ceramide bilayers were resistant to solubilization by Triton X-100 at 4 °C, the non-solubilized residue being enriched in ceramide.

5.3. Phase behavior and non-lamellar phase formation

The lamellar region of the phase diagrams of ceramide/phospholipid bilayers has been partially explored by different workers [75–77,79–81,84,95,96]. More detailed phase-composition diagrams for ceramide/phospholipid binary mixtures in excess water have been proposed for bovine brain ceramide/DPPC [73], C16:0 ceramide/POPC [78], C16:0 ceramide/DMPC [97], and C16:0 ceramide/DEPE [32]. In all these cases (with the exception of Silva et al., [78]), the phase diagrams have been constructed mainly on the basis of DSC data, supplemented by other techniques, e.g. X-ray scattering, ^{31}P-NMR, or electron microscopy.

The overall picture that arises, for ceramide concentrations up to ≈20 mol% (Fig. 5), is one in which, at low temperatures, in the gel phase, ceramide hardly mixes, if at all, with the phospholipid. Ceramide does mix with the phospholipid in the fluid phase, but non-ideally. As a result, a region in which various lamellar domains coexist appears at very low ceramide concentrations, and extends toward higher temperatures as ceramide concentrations increases. Only Silva et al. [78] explored the region of the phase diagram corresponding to high ceramide concentrations, and found a complex patchwork of different crystal and gel phases. Holopainen et al. [97] studied also phase transitions in the cooling mode, detecting very strong hysteresis in the thermal phase behavior of ceramide containing membranes.

Under equilibrium conditions, the disposition of lipids in cell membranes resembles that of the lamellar phases formed by some pure phospholipids – for example, egg phosphatidylcholine – in water. However, the idea that transient non-lamellar structures may form in membranes under nonequilibrium conditions is now widespread. Such non-lamellar structures are indeed formed under equilibrium conditions by certain phospholipids. One of them, dielaidoyl phosphatidylethanolamine (DEPE) exhibits a reversible lamellar-to-inverted hexagonal phase transition at 65 °C. Inverted hexagonal, together with inverted cubic phases, are believed to be involved in processes of membrane fusion and fission ([98,99], and references therein). Veiga et al. [84] have analyzed the lamellar-to-inverted hexagonal phase transition of DEPE, either pure or in mixtures with natural long-chain ceramides, by a combination of differential scanning calorimetry and ^{31}P-NMR spectroscopy. Ceramides decrease the transition temperature, without significantly modifying the transition enthalpy, thus facilitating the inverted hexagonal phase formation. Additionally, ^{31}P-NMR indicates that, under certain conditions, lamellar and hexagonal phases may coexist. Earlier studies by Ruiz-Argüello et al. [14] had shown, also by ^{31}P-NMR spectroscopy, that the presence of 10 mol% ceramide facilitated the thermotropic lamellar-to-"isotropic" (non-lamellar) transition in mixtures containing phospholipids and cholesterol. However, when mixed with the more

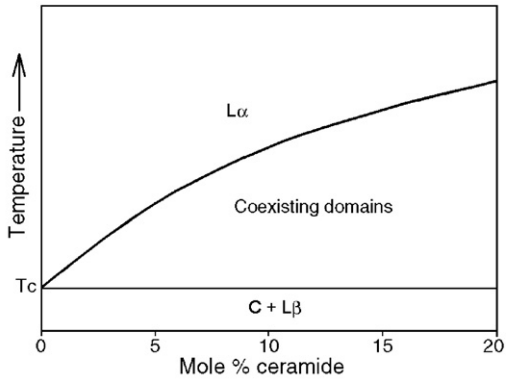

Fig. 5. Idealized temperature-composition diagram for a mixture of a phospholipid and a long-chain ceramide in excess water. T_c is the main gel–fluid transition of the pure phospholipid. C, ceramide-rich gel phase. L_β, phospholipid-rich gel phase. L_α, fluid phase.

bilayer-prone phosphatidylcholine/phosphatidylserine, long-chain ceramides are unable to induce non-lamellar phase formation even at high temperatures [83]. Yet in the latter system the addition of 15% dioleoylglycerol and 15% C6-ceramide leads to the partial conversion of the lipid into an isotropic phase, as detected by ^{2}H-NMR. More recently, using a combination of DSC, ^{31}P-NMR and X-ray scattering, Sot et al. [32] have explored systematically the non-lamellar (inverted hexagonal, or H_{II}) region of the C16:0 ceramide/DEPE phase diagram. As described above, ceramide was solubilized in fluid DEPE and had the effect of decreasing the lamellar–hexagonal (L–H) transition in a dose-dependent way (Fig. 6A). At ≈20 mol% ceramide the onset of the L–H transition overlapped with the completion of the gel–fluid transition at ≈48 °C, so that in this region of the phase diagram (>20 mol% ceramide) no pure fluid lamellar phase existed, and a direct gel-to-inverted hexagonal transition was possible. Long-chain ceramides have the propensity to stabilize inverted phase because of their geometry [100–102].

5.4. Membrane permeabilization by ceramides

A possible implication of ceramides in membrane permeability was already hinted at by Pascher in his early structural work [13]. Ruiz-Argüello et al. [14] prepared LUV composed of sphingomyelin, phosphatidylethanolamine and cholesterol at a 2:1:1 mol ratio, that contained entrapped fluorescent dyes in solution. Upon treatment with sphingomyelinase, ceramide was produced and leakage of vesicle aqueous contents occurred in parallel. (Simultaneous vesicle aggregation was also observed, see below). Leakage at various rates was also detected with other lipid compositions based on sphingomyelin and phosphatidylethanolamine, and, importantly, also with fluorescent dye-loaded and resealed erythrocyte ghosts after addition of sphingomyelinase. Moreover vesicle efflux has been detected, only in the presence of Ca^{2+}, as a result of the activity of a novel phospholipase C/sphingomyelinase, $PlcHR_2$ from *Pseudomonas aeruginosa* (L.R. Montes, unpublished). In further studies using the *Bacillus* sphingomyelinase, Montes et al. [103] found that ceramide formation in LUV loaded with fluorescein-derivatised dextrans gave rise to the release of dextrans of molecular mass ≈20 kDa, i.e. larger than cytochrome *c*. This is significant because ceramide may act physiologically releasing cytochrome *c* from mitochondria in the activation of apoptosis. The same authors compared vesicle efflux caused by sphingomyelinase and that caused by long-chain ceramides externally added to the pre-formed vesicles. Vesicle contents efflux was observed in both cases, although the same proportion of ceramides generated by sphingomyelinase induces faster and more extensive efflux than when added in organic solution to the preformed vesicles [103]. This may be related to the different morphologies of the ceramide-enriched domains when they are formed as a result of sphingomyelinase action or when they arise from ceramide pre-existing in the lipid mixture, being more branched, i.e. with a larger gel–fluid interface, in the former case [94].

The origin of leakage under the above conditions has not been fully explained. The addition or generation of ceramide in a pre-existing bilayer is required: neither ceramide incorporated into the lipid composition during vesicle preparation, nor the presence of sphingomyelinase in inactive form (e.g., in the absence of divalent cations), would induce leakage. The phenomenon may be related to the generation of "islets" or microdomains rich in ceramide. The ceramide-rich spots would constitute rigid, ordered microdomains in a "sea" of fluid lipids (for the tendency of ceramides to separate into domains, see

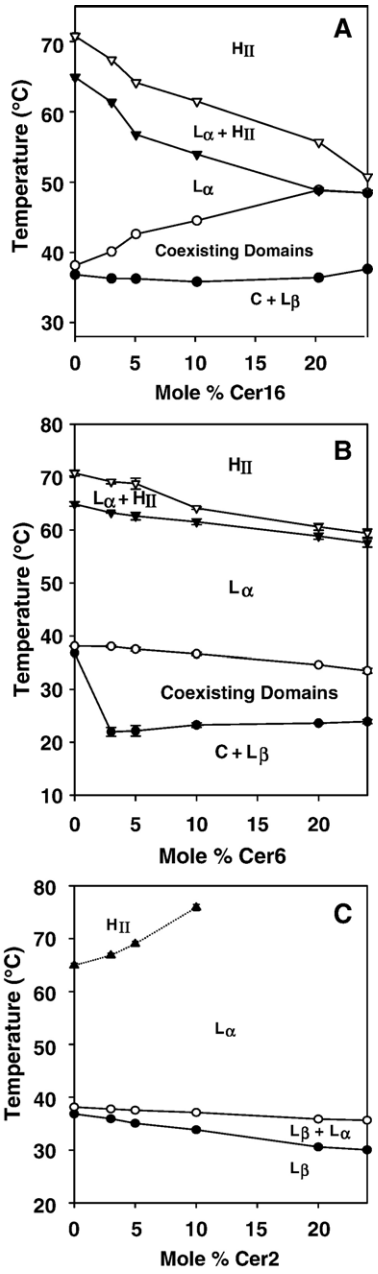

Fig. 6. Temperature-composition diagrams for (A) DEPE/Cer16, (B) DEPE/Cer6, (C) DEPE/Cer2 mixtures in excess water. Transition temperatures were derived from DSC thermograms. Phase structures were obtained from X-ray diffraction and ^{31}P-NMR, C, ceramide-rich gel phase. L_{β}, DEPE, or DEPE-rich, gel phase. L_{α}, liquid–crystalline, or fluid phase. H_{II}, inverted hexagonal phase. (●, ○) Respectively, onset and completion temperatures of the gel–fluid phase transition. (▼, ▽) Respectively, onset and completion temperatures of the lamellar–hexagonal phase transition. (Modified from [32]).

Domain formation, above). The situation of coexisting ordered and disordered domains in a bilayer is akin to the one prevailing at the T_c gel-to-fluid transition temperature of phosphatidylcholines. Vesicles composed of pure DMPC or DPPC are known to be particularly leaky at the T_c [104] and the interfaces between ceramide-rich and -poor regions would allow leakage in sphingomyelinase-treated bilayers. Structural defects induced by ceramide in phosphatidylcholine bilayers were observed by Zidovetzki et al. [105]. Alternatively, or additionally, generation of ceramide at one side of the membrane (sphingomyelinase was added to the preformed vesicles, so it is unlikely that it gets inside them) will give rise to membrane rearrangements including transbilayer ("flip-flop") movement of ceramide to the inner monolayer. In fact, the small polar headgroup of ceramide, as compared to sphingomyelin, facilitates its accommodation in the more highly-curved, inner monolayer. Transbilayer movements of lipids are likely to generate discontinuities in the membrane permeability barrier. Flip-flop movements of enzymically-generated ceramide have also been invoked by Holopainen et al. [76] to explain some of their results (see below the section devoted to ceramide-induced flip-flop).

It is interesting in this context that Colombini and co-workers [106], using mainly planar lipid bilayers and electrophysiological detection methods, observed ceramide-induced channel formation under conditions comparable to those used by Ruiz-Argüello et al. [14]. Later Siskind et al. [107] found that ceramide could actually increase the permeability of the mitochondrial outer membrane to cytochrome *c* and other small proteins. However, one debated aspect of their work is that they propose, as the mechanism for ceramide-induced efflux, the spontaneous formation of transmembrane channels composed of pure ceramide with ceramide chains parallel to the plane of the membrane. The structure, and even the existence of such channels has been challenged by other authors, although Anishkin et al. [108] have provided support to the transmembrane ceramide channels on the basis of molecular dynamics simulations. An additional aspect of the work by Colombini and co-workers is that dihydroceramide, in which the C4–C5 sphingosine bond is saturated, and is inactive in promoting apoptosis at the cellular level, is also little active in permeabilizing planar lipid bilayers [106], and in fact inhibits permeabilization by ceramides [109].

5.5. Ceramide and membrane fusion

Enzymatic generation of ceramides in sphingomyelin-containing large unilamellar vesicles leads to extensive vesicle aggregation, as detected by an increase in suspension turbidity, as well as by cryo-transmission electron microscopy of the vesicle suspensions [14,110]. These observations were confirmed by Holopainen et al. [76], who could also ascertain that, in contrast, ceramide-containing LUV (in which ceramide and phospholipids had been mixed in organic solvent prior to hydration and extrusion) did not show any signs of aggregation for up to 24 h of incubation. Ruiz-Argüello et al. [14] attributed aggregation to the localized, asymmetric generation of ceramide-rich patches on the vesicle surface through the action of the enzyme. The relative dehydration and exposure of hydrophobic surfaces at those patches would ensure aggregation, driven mainly by hydrophobic forces.

Ruiz-Argüello et al. [14], while detecting extensive aggregation, were unable to measure any intervesicular mixing of lipids, or of aqueous contents, that could have been correlated with vesicle–vesicle fusion. Giant vesicles, arising from multiple fusion events, were, however, detected by cryo-transmission electron microscopy under slightly different conditions, namely lower sphingomyelinase activity, by Basáñez et al. [110]. The latter fusion phenomenon has not been studied in more detail, but instead Ruiz-Argüello et al. [111] have described the cooperative action of ceramide and diacylglycerol, generated in situ respectively by sphingomyelinase and phospholipase C, to induce aggregation and fusion of LUV containing equimolar mixtures of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and cholesterol. Neither enzyme was able to produce aggregation or fusion on its own. Interestingly, microbial enzymes that are known to possess both phospholipase C and sphingomyelinase activities, have been shown to induce vesicle fusion. This is the case of the phospholipases C/sphingomyelinases of *Listeria monocytogenes* [112] and *Pseudomonas aeruginosa* (L.R. Montes, unpublished).

Moreover, Holopainen et al. [113] have shown microscopic images of "endocytotic" budding of vesicles composed of phosphatidylcholine and sphingomyelin, upon addition of sphingomyelinase. The authors attributed the observed budding to the tendency of ceramide to separate into domains, and to its negative spontaneous curvature, that leads to membrane invagination. It is clear, in summary, that ceramides can either induce by themselves, or with the help of other molecules, the promotion of vesicle aggregation and/or fusion.

At present, the most widely accepted mechanism for membrane fusion involves the transient participation of a non-lamellar structure, the "stalk", a semitoroidal lipidic structure that would allow the merger of the closest leaflets of apposed membranes. Ceramides, because of their molecular geometry, can be accommodated in these non-lamellar structures more easily than lipids with larger polar headgroups, such as sphingomyelin. A series of studies by Alonso, Goñi, and co-workers (reviewed in [30]), have established a correlation between non-lamellar phase formation and facilitation of membrane fusion. As discussed above, ceramides promote the transition from lamellar to non-lamellar structures, thus they could in the same way facilitate stalk formation and membrane fusion.

Most interesting with respect to the fusogenic capacities of ceramide observed in model systems is the data according to which endocytotic vesicles are formed in the absence of ATP when fibroblasts or macrophages are treated with exogenous sphingomyelinase or ceramides. Zha et al. [114] describe how the addition of bacterial sphingomyelinase to ATP-depleted fibroblasts or ceramides induces the rapid formation of vesicles, approximately 400 nm in diameter, not enriched in clathrin or caveolin, that pinch off from the plasma membrane and go into the cytosol. The authors speculate that hydrolysis of sphingomyelin on the plasma membrane causes inward curvature and subsequent formation of sealed vesicles. This is in full agreement with the biophysical data. While sphingomyelin is a lipid

promoting the stability of lipid bilayers, ceramide, because of its relatively small polar headgroup ("conical shape", in the nomenclature of Israelachvili et al. [101]) induces a "negative curvature" [100], that is, inward curvature of the outer monolayer of the plasmalemma. Higher ceramide concentrations, particularly if localized and asymmetric, will lead to vesicle fission by the mirror image mechanism of their facilitation of membrane fusion [84,111]. During preparation of this review, further evidence has been published of ceramide-induced membrane fusion in a cellular system, namely sea urchin cortical vesicles [114 bis].

5.6. Transmembrane (flip-flop) lipid motion

Studies by Bai and Pagano [115] using a fluorescent analog of ceramide have provided values for the half-times of flip-flop transbilayer diffusion of this lipid. The same study included data for the corresponding fluorescent analogs of phosphatidylcholine, sphingomyelin and diacylglycerol. Ceramide flip-flop rate had a $t_{1/2}$ of about 22 min, much faster than the phospholipids, but considerably slower than the diacylglycerol analog (70 ms).

A study published recently by López-Montero et al. [116] has studied ceramide flip-flop using a different approach. These authors observed that incorporation of a very small percentage of ceramides (approximately 0.1% total lipids) to the external leaflet of egg phosphatidylcholine GUV suffices to trigger a shape change from prolate to pear-shaped vesicle. By observing the reversibility of this shape change the transmembrane diffusion of lipids was inferred. López-Montero et al. found a half-time for unlabeled ceramide flip-flop of less than 1 min at 37 °C. The rapid diffusion of ceramides in a phosphatidylcholine bilayer was confirmed by flip-flop experiments with a spin-labeled ceramide analog incorporated into large unilamellar vesicles.

Related studies from our laboratory [117,118] have demonstrated, using very different procedures, that ceramides induce generalized flip-flop motion in model and cell membranes. In a first series of experiments [117], ceramide generation took place *in situ* through the action of a sphingomyelinase added to liposomes or cell membrane vesicles in suspension. Two different novel assays were developed to detect transbilayer movement. One of the assays required the preparation of vesicles containing a ganglioside only in the outer monolayer and entrapped neuraminidase. Sphingomyelinase activity induced ganglioside hydrolysis under conditions in which no neuraminidase was released from the vesicles. The second assay involved the preparation of liposomes or erythrocyte ghosts labeled with a fluorescent energy donor in their inner leaflets. Sphingomyelin hydrolysis was accompanied by fluorescence energy transfer to an impermeable acceptor in the outer aqueous medium.

In other experiments ceramide was added externally to pre-formed vesicles. Flip-flop motion was demonstrated either by the just mentioned fluorescence energy transfer assay, or by the method of Müller et al. [119], based on the excimer-forming capacity of the fluorescent probe pyrene, incorporated into phosphatidylcholine or phosphatidylserine analogs. When added in organic solution to pre-formed liposomes, egg ceramide induced transbilayer lipid motion in a dose-dependent way. Interestingly dihydroceramides, that are inactive from the point of view of most ceramide physiological activities, were also unable to promote flip-flop [118].

The origin of ceramide-induced transbilayer lipid movement is probably the result of two different phenomena. One is the well-known property of ceramide of facilitating the lamellar to non-lamellar lipid phase transitions. This does not mean that, under conditions of the experiments just described, ceramide would bring about a phase transition so that membrane lipids would be in the inverted hexagonal phase at the end of the experiment. Instead, ceramide at one side of the membrane would induce the transient formation of non-lamellar structural intermediates, which cause the loss of lipid asymmetry in the bilayer, i.e. the transbilayer movement of ceramide together with other lipids. The second underlying phenomenon would be the tendency towards mass conservation (or constancy of lateral tension) in each membrane leaflet. When one (in our case the outer) membrane monolayer becomes enriched in ceramides, they diffuse (through non-lamellar intermediates) toward the other leaflet. This is counterbalanced by lipid movement in the opposite direction, so that net mass transfer between monolayers is avoided.

The observation of ceramide-induced flip-flop movement may explain an otherwise mysterious aspect of ceramide signalling in cell physiology, namely that sphingomyelin, perhaps the main source of ceramide in this context, is predominantly located in the outer monolayer of the plasma membrane, whereas ceramide receptor proteins are intracellular. Sphingomyelin on the outer leaflet appears to be hydrolyzed by a secreted form of acid sphingomyelinase [89]. The resulting ceramide, originally generated in the outer leaflet, would soon distribute among both monolayers, thus being able to bind intracellular proteins docking the plasma membrane inner monolayer.

5.7. Ceramide-cholesterol interactions

In the previous sections we have concentrated on ceramide in bilayers composed almost always of a single phospholipid. We shall now turn our attention to the interactions of ceramide with cholesterol. The simplest relevant system is the ceramide/cholesterol monolayer. Scheffer et al. [120], using grazing incidence X-ray diffraction, have shown the existence of a crystalline mixed phase of the two components, within a range of compositions of ceramide/cholesterol between 0:100 and 33:67 mole ratios. Between 50:50 and 70:30, the mixed phase coexists with a ceramide crystalline phase. Above 70:30 mole ratio, only the latter phase is found. The authors conclude that the new crystalline mixed phase constitutes a stable, homogeneous arrangement of cholesterol with ceramide.

In a more complex system, namely sphingomyelin/ceramide/cholesterol bilayers, Massey [77] observed, on the basis of fluorescence spectroscopic methods, that the properties of the bilayers were dependent upon both the concentrations of

cholesterol (from 0 to 33 mol%) and of ceramide (from 0 to 10 mol%). In the fluid phase, both ceramide and cholesterol separately increased acyl chain order, and their effects were additive. More interestingly at 37 °C, near the gel–fluid transition of sphingomyelin, ceramide and cholesterol had opposite effects on acyl chain motion. Ceramide decreased acyl chain motion as a result of the formation of gel-like sphingomyelin/ceramide domains ("complexes" in the interpretation of Massey), while cholesterol increased acyl chain motion by decreasing the amount of gel phase lipid. The presence of 33 mol % cholesterol abolished the formation of gel phase sphingomyelin/ceramide "complexes" (domains). The presence of a single, continuous phase in mixtures of sphingomyelin, ceramide and cholesterol, with a (sphingomyelin + ceramide)/cholesterol 2:1 mole ratio was also hinted at in our studies of sphingomyelinase degradation of sphingomyelin/cholesterol bilayers [121].

An interesting observation has been made by London and co-workers [122,123] that ceramide displaces cholesterol from ordered lipid domains containing phosphatidylcholines (or sphingomyelin) and cholesterol. These authors, following a rather extended, if not rigorous, trend, identify fluid-ordered lipid domains with the transient membrane domains known as "rafts" [124]. Fluid ordered lipid domains are isolated by centrifugation as the non-solubilized residue ("detergent-resistant membrane") after Triton X-100 treatment. When part of the saturated phospholipids are replaced by ceramide in the original vesicles, the detergent-resistant ordered domains are found to contain ceramide instead of cholesterol.

Tight lipid packing is critical for sterol displacement by ceramide. The authors suggest that the driving force of the event is the hydrophobic effect that tends to minimise unfavourable contact between the hydrocarbon groups of the small headgroup lipids (ceramides, sterols) and the surrounding aqueous environment. Hydrocarbon-water contact would be prevented in ordered domains because of the capacity of certain lipids with large headgroups (sphingomyelin, phosphatidylcholines) to accommodate small headgroup lipids in the ordered domain lattice. Ceramide would replace sterols with advantage from this point of view. (See in this respect the "molecular cavity" concept developed for ganglioside/ceramide mixtures by Carrer and Maggio, [125]).

The results by Megha and London [122] have been obtained with the use of detergents, with the implicit assumption that membrane domains recovered in remnants after detergent treatment correspond to structures that existed already in the membranes before detergent addition. This assumption has been criticized elsewhere [126]. However, a number of data are available that support Megha and London's contention: (i) Slotte and co-workers [127,128] and Chiantia et al. [87] showed cholesterol displacement by ceramides using a detergent-free procedure; (ii) Yu et al. [129] found essentially the same effect using detergent but in the more complex, and relevant, system of mammalian cells; (iii) as mentioned by Megha and London themselves, a phenomenon of cholesterol displacement from plasma membranes induced by sphingomyelinase activity had been reported [130,131].

5.8. Short-chain ceramides

Ceramides with an N-acyl chain shorter than 8–10 C atoms are usually called "short-chain ceramides". Since most naturally occurring ceramides cannot be dispersed in water—because of their hydrophobicity—short-chain ceramides, and particularly N-acetylceramide, or C2-ceramide, have been extensively used as agonists when ceramide effects had to be elicited, both in intact cells and in cell-free systems. Short-chain ceramides form clear dispersions in water, although the nature of the dispersion, or of the putative molecular aggregates, have remained unexplored until recently. In the nomenclature of Small [65], long chain ceramides are "insoluble non-swelling amphiphiles," while short-chain ones are "insoluble swelling amphiphiles." In fact, the use of the N-acetyl derivative is not devoid of a certain scientific pedigree. C2-ceramide is the sphingolipid equivalent of 1-oleoyl-2-acetyl-*sn*-glycerol, used by Nishizuka [132] precisely because of its solubility properties. But just because of these physical properties, so different from those of the "natural compound", the use of C2-ceramide has been the object of criticism. Some recent work from ours and other laboratories has been devoted to compare the biophysical properties of ceramides with different N-acyl chain length. A summary of some of these studies may be found in Goñi et al. [133].

From the structural point of view, the NMR characterization of ceramide structure in solution by Li et al. [25] included a comparison of C2 and C18 ceramides. In both cases the conformation of the polar moiety of the molecule was the same, including the characteristic H-bond network between NH protons and OH groups, that was not found in e.g. dihydroceramide. The maintenance of the headgroup conformation irrespective of N-acyl chain length explains that C2 ceramides reproduce most of the long-chain ceramide signalling effects, because their interaction with the receptor enzymes occur probably through the polar head.

A systematic comparative study of the membrane properties of short- and long-chain ceramides has been carried out by Sot et al. [31,32]. Using monolayer techniques, these authors found that C16 ceramide behaves indeed as an insoluble, non-swelling amphiphile, not partitioning into the air–water interface, thus not modifying for hours the surface pressure of the aqueous solutions into which it is included, as mentioned above. By contrast both C2 and C6 ceramides behave as soluble amphiphiles, up to approx. 100 μM, beyond which they give rise to large aggregates and precipitate. At low (micromolar) concentrations, they become oriented at the air–water interface, increasing surface pressure in a dose-dependent way up to ca. 5 μM bulk concentration. At higher concentrations, the excess ceramide forms micelles, critical micellar concentrations of both Cer2 and Cer6 being in the 5–6 μM range [31].

When the air–water interface is occupied by a phospholipid, C2 and C6 ceramides, but not C16 ceramide, injected into the subphase, become inserted in the phospholipid monolayer, causing a further increase in surface pressure. Both short-chain ceramides become inserted in phospholipid monolayers with initial surface pressures above 40 mN/m, which ensures their capacity to become inserted into cell membranes whose

monolayers are estimated to support an average surface pressure of about 30 mN/m. C2 and C6 ceramides, unlike their C16 homologue, had detergent-like properties, such as giving rise to phospholipid–ceramide mixed micelles when added to phospholipid monolayers and bilayers [31]. Previous studies by Simon and Gear [67] and by Di Paola et al. [36] described some properties of short-chain ceramides that were not shared by long-chain ceramides, in particular the ability of the former to perturb cell membranes. The results by Sot et al. [31] provide a physical basis for the observed different behavior.

In a different series of studies, Sot et al. [32] compared the phase behavior of C2, C6 and C16 ceramides in mixtures with DEPE. Combining DSC, X-ray scattering and ^{31}P-NMR these authors could build up phase diagrams for the three mixtures (Fig. 6). The main differences between the long- and short-chain ceramides are those of miscibility with the phospholipid, and of geometrical shape. As discussed above, long-chain ceramides are immiscible with phospholipids in the gel phase, while short-chain ceramides mix quite well. The final outcome is that C16 ceramide increases the mid-point temperature of the gel–fluid transition, and gives rise to ceramide-rich and -poor coexisting domains, while C2 ceramide lowers the gel–fluid transition temperature and permits only a narrow area of domain coexistence. The geometry of long-chain ceramides is such that they favour "negative curvature" of the monolayers (by convention, the outer monolayer of a cell plasma membrane has a positive curvature), thus they facilitate the lamellar-to-hexagonal phase transition. C2 ceramide has a different geometry, that favours "positive curvature", thus it opposes DEPE tendency to form inverted hexagonal phases above 65 °C [32]. C6 ceramide displays intermediate properties between the other two: it lowers both the gel–fluid and the lamellar–hexagonal transition temperatures of the phospholipid.

Other ceramide properties are influenced by N-acyl chain length. In our studies of ceramide-induced solute efflux [103], the capacity of the various ceramides to induce release of vesicle contents decreased in the order C16>C6>>C2. However, several authors have described that C2 ceramide has the ability to break down the membrane permeability barrier [36,67,106]. In fact, Simon and Gear [67] observed actual lysis of platelets at ceramide/lipid mole ratios of 0.5 (this value was never higher than 0.1 in Montes et al. [103]). The observation by Simons and Gear corresponds most probably to the detergent properties of C2 ceramide, put forward by Sot et al. [31]. Di Paola et al. [36] show that C2 ceramide can dissipate the inner mitochondrial membrane potential, while Siskind and Colombini [106], using electrophysiological methods, detect pore formation in planar bilayers. In the latter two cases the observed phenomenon is a transient increase in membrane conductivity and its measurement has an inherent greater sensitivity than that of liposomal release of contents. Thus it may well be that permeabilization phenomena caused by C2 ceramide are so small, in comparison with those caused by C16-ceramide, that they become below the level of detection of fluorescence spectroscopy, but not of electric measurements. Siskind et al. [134] have studied in detail C2 ceramide-dependent changes in membrane conductance.

N-acyl chain length also appears to be important for flip-flop lipid motion. C6 and C2 were less active than egg ceramide in promoting transbilayer lipid transfer [118]. This would be in agreement with the long-chain (but not the short-chain) ceramide propensity to induce inverted hexagonal phases as the origin of flip-flop movements [32]. Finally short-chain ceramides, as opposed to long-chain ones, do not give rise to detergent-resistant membranes in mixtures with sphingomyelin [95]; nor can they displace cholesterol in liquid ordered domains [128], probably as a result of their failure to form highly packed arrangements with saturated phospholipids, or sphingomyelin. What is the frontier between the long- and short-chain ceramides? In the absence of systematic studies, the available data suggest that it is close to the N-octyl chain. Carrer et al. [96,135] have described a phase diagram for DMPC/C8 ceramide mixtures and it resembles more the lamellar region of the DEPE/C16 ceramide than that of the DEPE/C2 ceramide mixture. In turn Nybond et al [128], studying the ability of ceramides to displace sterols form liquid-ordered domains (see previous section on ceramide-cholesterol interactions), found that an N-acyl chain with at least 8 methylene units was required for a ceramide to displace cholesterol. Carrer et al. [96,135] made the interesting observation that, in DMPC/C8 ceramide mixtures, ceramide-enriched domains may exhibit partial interdigitation of ceramide chains in the gel phase, producing a correlation between the organization of both leaflets. Interdigitation is often linked to acyl chain asymmetry [136]. It will be worth exploring whether interdigitation occurs with ceramides of different N-acyl chain length, and with phospholipids with acyl chains longer than C14.

In view of the observed differences between short- and long-chain differences, it should be asked whether short-chain ceramides are acceptable as experimental analogs of their long-chain counterparts. Our point of view has not changed much from year 2000, when this matter was discussed at length in Kolesnick et al. [5]. The use of C2-ceramide as an experimental tool when an agonist is required to elicit a ceramide-dependent response continues to be justified. "Water soluble" ceramides may overcome the membrane barrier and, because they possess the appropriate stereochemistry, they will bind specific sites in the target proteins. The differences in physical properties, mainly swelling behavior, between long- and short-chain ceramides can explain several instances in which, for example, sphingomyelinase and cell-permeable ceramides have lead to different biological effects [137], or those cases where long- and short-chain ceramides have produced diverging effects [35,138]. However, the fact that occasional differences with the long-chain compounds are detected should not preclude the use of C2-ceramide. Rather, observation of such differences could provide additional clues on the mechanism of action of ceramides at that particular level.

6. Other ceramides, and ceramide derivatives

Only a few, scattered data are available for molecules structurally related to the sphingosine-based ceramides. It has been mentioned in the section on ceramide structures that

dihydroceramides, i.e. containing a sphingosine with a saturated C4–C5 bond, are physiologically inactive, and they exhibit impaired properties of H-bonding and hydration [25] and very different interfacial properties, including looser packing [29]. Ceramides containing the C4–C5 double bond but in the cis (non-physiological) configuration are quite different from the point of view of the internal network of H-bonds (M.C. Yappert, unpublished results), they insert more easily than the trans-homologues in lipid monolayers, but they are less effective in inducing flip-flop motion (F.X. Contreras, unpublished results).

The hydration and mesomorphic properties of N-(α-hydroxyacyl)-sphingosine, a kind of ceramide that occurs in the stratum corneum of the skin, have been explored by Raudenkolb et al. [139]. The hydrated form of this lipid has been found to exist in a variety of phases, depending on the thermal history of the sample. The system can only be hydrated by heating the lipid above the melting chain transition temperature. Above this temperature, the hydroxyacyl ceramide forms a hexagonal phase.

An interesting synthetic (non-natural) ceramide derivative with a net positive electric charge has been prepared [140] and made commercially available. Positively charged ceramides increased inner mitochondrial membrane permeability, and triggered release of cytochrome *c*, although these effects appear to be due to activation of specific ion transporters, rather than to a generalized loss of bilayer barrier functions.

Ceramide-1-phosphate is emerging as a novel bioactive sphingolipid (see companion review in this Special Issue [141]). Ceramide-1-phosphate has a variety of cell actions that appear to be cell type-specific, e.g. it is mitogenic for fibroblasts, but rather appears to control phagocytosis in neutrophils [142]. Ceramide kinase, the enzyme responsible for the synthesis of ceramide-1-phosphate, translocates during activation of transfected COS-1 phagocytic cells from the cytosol to a lipid "raft" fraction [143]. These authors measured the acyl chain order in the plasma membrane using Laurdan fluorescence, and found that transfected cells showed a higher degree of order than control cells after stimulation. An increased order should favour membrane fusion at the sites of phagocytosis [144]. Work in progress in our laboratory suggests that ceramide-1-phosphate has also in common with ceramide a marked tendency to facilitate the transition from lamellar to inverted hexagonal phases (J. Sot, unpublished).

7. The relevance of biophysical studies in cell biology

The question on how relevant biophysical studies, often carried out on model systems, are to cell physiology and pathology is one that worries both biophysicists and cell biologists. It is, in fact, a question on methodology and, ultimately, a question on how science is done, i.e. on the philosophy of science. The question on the philosophy is indeed a question on the validity of the reductionist approach. Biophysics, or biophysical chemistry, admittedly takes to a limit the reductionist approach in biology. We operate as if biology were nothing but chemistry, and physical chemistry at that. But it has to be agreed that reductionism, as a heuristic tool, is at the very essence of molecular biology, and there is no reason to dismiss it at this point.

From a more down-to-earth point of view, the validity of biophysics as a methodology in the cell sciences should not be given a definitive, general reply. Methods are good as long as they are not proven wrong, and all of them should be permanently questioned. The biophysical approach to cell biology is no exception. Many important things that we know about cell membranes have been first observed, or explained, in the model membranes: monolayers, bilayers, liposomes … (Equally, some dubious concepts have also originated in the biophysical field). Thus a healthy dose of skepticism will undoubtedly benefit biophysics … as it will cell biology.

More specifically, membrane biophysical studies are often criticized on the basis of the unrealistic lipid compositions employed. What is the meaning of preparing liposomes (or monolayers, etc.) containing e.g. 15 mol% ceramide, when ceramide contents in cell membranes is never that high? The answer is that the experiment is a sort of magnifying glass applied to the "real" biological system. High ceramide (or sphingosine, etc.) concentrations do occur in cell membranes, only localized in space and time, i.e. in transient microdomains. In order to understand the properties of these short-lived membrane patches, we must reproduce them at a larger scale, in space and time. In order to be able to detect a phenomenon, we must have it multiplied. With many biophysical techniques, a single membrane fusion event taking place in a cell or vesicle population would go undetected. In a typical experimental set-up, a veritable orgy of fusion events is induced, in order to obtain average data of a good number of them in a reasonable time.

In the field of lipid second messengers, the anomaly has been pointed out that lipids stay usually in the membranes, while target proteins are known that are totally "soluble". This matter has been dealt with in Kolesnick et al. [5], and specifically in the review by van Blitterswijk et al. [145]. Should these lipids be considered as second messengers or as modulators of membrane structure and dynamics? The answer is, certainly, both. Some lipid second messengers, of which ceramide is a prime example, are so hydrophobic that their cytosol concentration is negligible. Even in this case, the possibilities of exerting physiologically meaningful effects are manyfold: (i) lipid-induced changes in the membrane physical properties (e.g. lipid chain order) may influence the activity of intrinsic enzyme proteins; (ii) other physical changes (e.g. permeabilization) may induce local changes in ion concentrations, in turn affecting cytosolic enzymes in the vicinity; (iii) proteins that are isolated as soluble enzymes may in fact dock transiently to membranes, thus binding signalling lipids; (iv) lipid transfer proteins (e.g. the ceramide transfer protein) may bring very hydrophobic lipids to targets that are distant from the membranes of origin. Several examples of these possibilities can be found in the reviews mentioned above in the paragraph.

8. Summary

(1) Sphingosine, (2S, 3R, 4E)-2-amino-4octadecen-1,3-diol, and ceramide (N-acylsphingosine) are the simplest sphingolipids. They constitute the basic structure of sphingomyelin, cerebrosides, gangliosides, and other complex

sphingoglycolipids. In addition, sphingosine, ceramide, and its phosphorylated derivatives are important metabolic regulators.

(2) The simple sphingolipids, ceramide in particular, are very hydrophobic. They can exert their physiological effects either through changes in membrane properties or else through binding specific target proteins that dock more or less transiently the membrane bilayer.

(3) Sphingosine is one of the rare lipids with a net positive charge at pH 7.4. This enables it to interact with important negatively charged phospholipids like phosphatidylserine, cardiolipin, or the phosphoinositides. In addition, sphingosine rigidifies the bilayer lipid acyl chains and, probably as a consequence of the latter property, it may permeabilize membranes with coexisting domains of different fluidities.

(4) Ceramides cause a variety of important changes in the physical properties of the cell membranes: they increase the order of the acyl chains, cause lateral separation of domains enriched in ceramide, permeabilize lipid bilayers, facilitate membrane fusion and fission, and induce transmembrane (flip-flop) lipid motion.

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