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Unique thermal behavior of sphingomyelin species with nonhydroxy and 2-hydroxy very-long-chain (C28-C32) PUFAs[S]

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Abstract **In rat germ cells and spermatozoa, sphingomyelin (SM) contains molecular species with nonhydroxy (n) and 2-hydroxy (h) very-long-chain polyunsaturated fatty acids (V), the most abundant being SMs with (n- and h-) 28:4n-6, 30:5n-6, and 32:5n-6 as acyl chains. The aim of this study was to gain information about their thermotropic behavior and interactions with other lipids. After isolation from rat testis, multilamellar and giant unilamellar vesicles from these SMs were examined using fluorescent probes. Only n-32:5 SM and h-32:5 SM displayed a gel-liquid transition temperature (Tt ~ 21–22°C), the rest remaining in the liquid state in the 5°C–45°C range. The degree of order was larger in bilayers of any of the h-V SMs than in those of their chain-matched n-V SMs. Both, but n-V SM relatively more than h-V SM, decreased the Tt of dimyristoylphosphatidylcholine as their proportion increased in binary phosphatidylcholine:SM liposomes. In contrast to the established ability of 16:0 SM to form lateral cholesterol/SM-rich ordered domains in ternary dioleoylphosphatidylcholine: cholesterol:SM bilayers, neither n-V SM nor h-V SM showed a tendency to do so.[F] Thus, these SMs are in the fluid state and are not involved in this type of domains in spermatozoa at physiological temperatures. However, this state could be altered at the very low temperatures at which these gametes are usually preserved.**—Peñalva,D. A., N. E. Furland, G. H. López, M. I. Aveldaño, and S. S. Antollini. **Unique thermal behavior of sphingomyelin species with nonhydroxy and 2-hydroxy very-long-chain (C28-C32) PUFAs. *J. Lipid Res.* 2013.** 54: **2225–2235.**

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Sphingomyelin (SM) is a ubiquitous sphingolipid component of cell membranes in vertebrate tissues. Like phosphatidylcholine (PC), it has phosphorylcholine as the head group, its hydrophobic moieties being a long-chain or a very-long-chain fatty acid amide-bound to the long-chain base sphingosine. Molecular species naturally occurring in mammalian SM often contain saturated (16:0, 18:0, 24:0) or monounsaturated (18:1, 22:1, 24:1) fatty acids (1).

The testis and spermatozoa of various mammals including man contain SM species with an infrequent series of very-long-chain polyunsaturated fatty acids (VLCPUFAs) (2). These are elongated versions of ordinary 20- or 22-carbon atom tetraenoic, pentaenoic, and hexaenoic fatty acids of the n-3 or the n-6 series, according to the mammalian species. Whereas SMs from bovine and ovine spermatozoa are exceedingly rich in n-3 VLCPUFA with up to 34 carbon atoms, those from rodent germ and sperm cells mostly contain tetraenoic and pentaenoic fatty acids of the n-6 series with 28 to 32 carbon atoms, representative components being 28:4n-6, 30:5n-6, and 32:5n-6 (2, 3).

In some mammals including the rat, a unique series of molecular species of SM containing 2-hydroxylated derivatives of the mentioned VLCPUFA also occurs in testis and spermatozoa (4). In adult rat testis, the SM species having nonhydroxy VLCPUFA and 2-hydroxy VLCPUFA (n-V SM and h-V SM, respectively) are exclusive components of spermatogenic cells, as are the corresponding ceramides containing

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; DPH, 1,6-diphenyl-1,3,6-hexatriene; *EF*, emission factor; emGP, emission generalized polarization; exGP, excitation generalized polarization; GP, general polarization; GPL, glycerophospholipid; GUV, giant unilamellar vesicle; h-V SM, SM species with 2-hydroxy very-long-chain PUFA; n-V, SM species with nonhydroxy VLCPUFA; PC, phosphatidylcholine; S+M SM, SM with saturated (S) and monoenoic (M) fatty acid; Tt, transition temperature; VLCPUFA, very-long-chain PUFA.

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these fatty acids (5, 6). Both types of SM species are absent altogether from the testis before puberty, n-V SM appearing before h-V SM after the onset of spermatogenesis (6). This is due to the fact that spermatocytes appear before spermatids after this onset, and the former cells exclusively contain n-V SMs whereas the latter, as do spermatozoa, contain more h-V SM than n-V SM (7). Further work showed that both groups of SM species end up exclusively located on the heads rather than the tails of rat spermatozoa (8).

Many questions about the properties and functions of these unique molecular species of SM remain to be investigated. The aim of this study was to explore some of their biophysical characteristics and modes of interaction with other lipids in model membrane systems. To this end, it was first necessary to surmount the difficulty of separating the n-V SM and h-V SM species from rat testis, between themselves and from the most abundant (nonhydroxy) saturated and monoenoic fatty acid-containing SM species (S+M SM). A combination of adsorption TLC techniques was applied to recover these three subfractions in purified form. Part of the studies required individual molecular species of n-V SM and h-V SM, which were obtained by subjecting each of these subfractions to HPLC.

In liposomes, the thermotropic properties of the unique SM subfractions or molecular species typical of germ or sperm cells were evaluated following different strategies of fluorescence spectroscopy. Two amphiphilic fluorescent probes widely used to study bilayer structure and changes in phase states of their lipids (9–12), namely 6-dodecanoyl-2-(dimethylamino) naphthalene (Laurdan) and 1,6-diphenyl-1,3,6-hexatriene (DPH), were used. These tools allowed us to evaluate the influence of the 2-hydroxyl group, the number of double bonds, and the fatty acid chain length on the properties of these lipids. The effects produced by these SMs when added to liposomes composed of a glycerophospholipid (GPL) whose thermotropic behavior is well characterized, as is the case of dimyristoylphosphatidylcholine (DMPC), were also analyzed. Finally, in multilamellar and giant unilamellar vesicles (MLVs and GUVs) composed of ternary systems containing dioleoylphosphatidycholine (DOPC), cholesterol, and SM, the ability of n-V SM and h-V SM to promote the formation of ordered membrane domains was also studied. Our results show that the species under study display biophysical properties and behavior which could be considered exceptional in comparison with those of more ubiquitous SMs having long-chain and very-long-chain saturated and monoenoic fatty acids.

MATERIALS AND METHODS

Materials

Fluorescent probes from Molecular Probes (Eugene, OR) and phospholipids (DMPC, DOPC) from Avanti Polar Lipids (Alabaster, AL) were used without further purification. All solvents used in this study were HPLC grade (JT Baker, USA; Dorwill, Argentina). The GC and HPLC equipments were from Varian-Agilent, USA. A model 4800 SLM spectrofluorimeter (SLM Instruments, Urbana, IL) was used for fluorimetric measurements.

Male adult Wistar rats of different ages, with ad libitum access to a standard diet and water, treated and manipulated in accordance with the guidelines issued by the National Institutes of Health, USA, were used as the biological source of the SM species of this study. Immediately after rat sacrifice, the testes were removed, decapsulated, devoid of visible blood vessels, and immediately subjected to lipid extraction.

Lipid isolation and analysis

Lipid extracts were prepared from several testes using chloroform:methanol mixtures (13), taken to dryness under N_2, and resuspended in chloroform:methanol. Aliquots were taken to determine the content of total lipid phosphorus (14).

Preparative isolation of SM. Rat testis SM was separated from major GPL by TLC and recovered from the silica support by thoroughly eluting it with water:methanol:chloroform, 1:5:5 by vol, collecting the eluates and partitioning them by adding 4.5 vols of water (13). For TLC, lipid extracts were spotted on 500 μm-thick 20 × 20 cm glass plates previously prepared with silica gel H and a 3% magnesium acetate solution using a solvent mixture of chloroform:methanol:water (65:25:5, by vol). The eluted SMs were routinely taken to dryness and exposed to a brief alkaline treatment in order to remove any potential lipid contaminant with ester-bound fatty acids (6, 7). By spotting an amount of lipid extracts equivalent to not more than about 1 mg total lipid phosphorus, rat testis SM tended to resolve into two bands. The one with the lower Rf value contained the h-V SM species contaminated by low proportions of nonhydroxy-SMs, mostly 16:0 and 18:0 SM. The fraction migrating closely ahead collected the bulk of SMs with nonhydroxy fatty acids. This included the n-V SMs of our interest plus a large proportion of species with saturated fatty acids (mostly 16:0 and 18:0) and also minor amounts of saturated and monoenoic fatty acids with longer chains. Their abundance was indeed a complication during the preparative isolation of SM species with both types of VLCPUFA.

This problem was circumvented by taking advantage of the high degree of unsaturation of the SM species with VLCPUFA. The whole SM from rat testis was first isolated and subsequently subjected to argentation TLC. By using plates previously prepared with 10 g silica gel G and 2 g $AgNO_3$ in water, drying, and running the same solvent, the species with saturated and monoenoic fatty acids migrated ahead whereas those with VLCPUFA lagged well behind. The latter were recovered together from these plates and subsequently exposed again to adsorption TLC on silica gel H as described above. Though laborious and time-consuming, this procedure resolved the species of SM with VLCPUFA into two subfractions, one rich in n-V SM and the other rich in h-V SM species. The fraction containing the S+M SM species was also collected for comparative purposes.

Molecular species of SM. The major individual molecular species of SM were separated by subjecting the n-V SM and h-V SM subfractions to reverse-phase HPLC. Chromatography was performed at 40°C using a stainless steel column (250 × 4.6 mm) packed with 5 μm spherical particles covered with octylsilane (C8) (Microsorb-MV, Rainin LC). The solvent was methanol:3 mM potassium phosphate buffer (pH 7.4) in a 96:4 (v/v) ratio, pumped isocratically at a flow rate of 1 ml/min. The SM subfractions recovered after TLC separation as described above were filtered to remove traces of particulate matter, dried, and redissolved in mobile phase. Aliquots containing not more than 5 μg of SM phosphorus were repeatedly injected to collect the amounts of the major SM species required for each analysis.

The HPLC peaks were detected with a variable-wavelength UV spectrophotometer placed at 203 nm. After collection, the SM

species were recovered by partition of the methanolic eluates with chloroform and water (13) and by evaporating to dryness the solvent obtained in the lower phase. Aliquots from each of the major fractions collected were reinjected under the same conditions in the HPLC to check their purity, which was over 95% for each isolated peak. The SM species were then quantified by their phosphorus content and their identity was confirmed by GC analysis of their fatty acids.

For GC, dried samples of SM were dissolved in N_2-saturated anhydrous methanol containing 0.5 N H_2SO_4 to convert their fatty acids into fatty acid methyl esters (FAMEs). In tubes with Teflon™-lined caps, the samples under N_2 were warmed at 45°C overnight. Prior to GC, all FAMEs were isolated by TLC using silica gel G plates precleaned with methanol:ethyl ether (75:25, v/v) and dried. The h- and n- FAMEs were separated conveniently by running hexane:ether (80:20, v/v) up to the middle of the plates and hexane:ether (95:5, by vol) up to the front. After elution, n-FAMEs were analyzed directly by GC and h-FAME after being converted into O-trimethyl-silyl derivatives (6). The equipment and conditions used for GC fatty acid analysis were described in previous work (5–7).

Liposome preparation

For fluorescence measurements, MLVs of the SM subfractions or species under study, alone or in combination with different proportions of other lipids, were prepared. Appropriate aliquots of the fluorescent probes Laurdan or DPH, dissolved in ethanol or dimethyl sulfoxide, respectively, were added to reach a 100:1 lipid-fluorescent probe molar ratio. The mixtures were placed for 1 h in the dark under a constant flow of N_2 for solvent evaporation. The dried lipid films were then hydrated by adding buffer A (20 mM HEPES buffer, 150 mM NaCl, and 0.25 mM $MgCl_2$, pH 7.4), vortexed for 1 min, and sonicated in an ultrasonic bath for 30 min. The temperature was kept above the gel-to-liquid crystalline phase transition temperature (Tt) of the lipid with the highest melting temperature. Each sample was diluted with buffer A to reach a final lipid concentration of 100 μM.

Fluorescence measurements

All fluorimetric measurements were performed using the vertically polarized light beam from a Hannovia 200 W Hg/Xe arc obtained with a Glan-Thompson polarizer (4 nm excitation and emission slits) and 5 × 5 mm quartz cuvettes. Emission spectra were corrected for wavelength-dependent distortions. Temperature was set with a thermostatted circulating water bath and liposomes were allowed to equilibrate at each temperature for 10 min before measurements.

Laurdan measurements. Excitation generalized polarization (exGP) (11, 12) was calculated according to the expression:

$$exGP = \frac{I_{434} - I_{490}}{I_{434} + I_{490}} \qquad Eq.\ 1$$

where I_{434} and I_{490} are the emission intensities at the characteristic wavelength of the gel phase (434 nm) and the liquid-crystalline phase (490 nm), respectively. The exGP values were obtained from the emission spectra at different excitation wavelengths (320–410 nm) or at one specific excitation wavelength (360 nm). Generalized polarization (GP) values correspond to exGP data, higher values denoting higher structural lipid order.

Emission GP (emGP) was calculated as follows:

$$emGP = \frac{I_{410} - I_{340}}{I_{410} + I_{340}} \qquad Eq.\ 2$$

where I_{410} and I_{340} are the excitation intensities at the wavelengths corresponding to the gel (410 nm) and the liquid-crystalline (340 nm) phases, respectively (15). The emGP values were obtained from the excitation spectra at different emission wavelengths (420–500 nm).

Anisotropy measurements. Fluorescence anisotropy was measured in the T format with Schott KV418 filters in the emission channels and corrected for optical distortions and background signals. The excitation and emission wavelengths used were 365 and 425 nm, respectively. The anisotropy value, *r*, was obtained according to the following equation (16):

$$r = \frac{\left(\frac{I_v}{I_h}\right)_v - \left(\frac{I_v}{I_h}\right)_h}{\left(\frac{I_v}{I_h}\right)_v + 2\left(\frac{I_v}{I_h}\right)_h} \qquad Eq.\ 3$$

associating the ratios of the intensities of emitted vertical (v) or horizontally (h) polarized light to the exciting vertical or horizontally polarized light, respectively (17).

Data analysis. Tts were obtained by calculating the second derivative of the experimental values (GP or *r*) as a function of temperature. The Tt was considered to correspond to a second derivative value of zero.

Fluorescence quenching. In order to compare n-V SM and h-V SM with 16:0 SM in their ability to form ordered domains in mixtures with GPL and cholesterol, the protocol described by Xu and London (18) was used. Multilamellar vesicles containing 100 μM total lipid and 1 mol % DPH as the fluorescent probe were prepared. Similar liposomes containing no DPH were prepared in order to deduct signal background. The lipid mixture consisted of DOPC, cholesterol, and each of the three SMs under study in the presence or in the absence of the fluorescence quencher lipid 1-palmitoyl-2-(10-doxyl)stearoylphosphatidylcholine (10-SLPC), in the proportions (%) found in **Table 1**.

Emission spectra of DPH as a function of temperature were obtained. Fluorescence excitation and emission intensities were 350 and 427 nm, respectively. Extinction of DPH fluorescence by 10-SLPC was expressed by an emission factor (*EF*), according to the following formula:

$$EF = \left(F/F_0\right)_{SM} - \left(F/F_0\right)_{DOPC} \qquad Eq.\ 4$$

where F and F_0 are the emission fluorescence intensities of DPH at each temperature with liposomes prepared with SM (or DOPC) in the presence or in the absence of the 10-SLPC quencher, respectively.

GUVs preparation and confocal fluorescence microscopy

In order to evaluate the formation of ordered lipid domains in membranes, the fluorescence probe DiI, which has a high preference for disordered domains, was included in GUVs containing DOPC, cholesterol, and SM in a 1:1:1 molar ratio. GUVs containing

TABLE 1. Lipid composition (%) of the four MLV conditions for *EF* calculation

	DOPC	10-SLPC	Chol	SM
$(F_0)_{DOPC}$	85.0	—	15	—
$(F)_{DOPC}$	42.5	42.5	15	—
$(F_0)_{SM}$	42.5	—	15	42.5
$(F)_{SM}$	—	42.5	15	42.5

either 16:0 SM, n-V SM or h-V SM were prepared by electroformation (19, 20). GUVs formation was performed at 45°C and they were observed at room temperature using confocal fluorescence microscopy. Instrumentation and procedures are described in supplementary Fig. II.

Statistical analysis

Intergroup comparisons were carried out using the paired Student's *t*-test. Comparison between two data lines was performed by ANCOVA (covariance analysis) test. Prior to this analysis it was ensured that the variance in the two groups of data to be compared was the same. The standard null hypothesis states that for two data lines to be the same, their slopes must be equal. Otherwise, the lines are different. Here, the null hypothesis was rejected with a p value < 0.05. When slopes were found to be the same, a further investigation of differences in the means was carried out using the test for equal intercepts. The Box Cox linearity transformation was applied when data were nonlinear, which provides a convenient way to find a suitable transformation that improves the fit.

RESULTS

Pure SM bilayers

Total SM from whole rat testis is composed of about 10% n-VLCPUFA and 15% h-VLCPUFA (5, 6). Saturated (S) followed by some monoenoic (M) fatty acids are major SM acyl groups. The three subfractions of SM separated after the combination of TLC procedures described in Materials and Methods, here abbreviated as n-V SM, h-V SM and S+M SM, respectively, typically had the fatty acid composition shown in **Table 2**. In each of the SM fractions, the sum of the depicted fatty acids represented more than 85% of the total fatty acids. Two major fatty acids were representative of each fraction: n-28:4 SM followed by n-30:5 SM in the n-V SM group, h-30:5 SM followed by h-28:4 SM in the h-V SM group, and 16:0 SM followed by 18:0 SM in the S+M SM group.

In order to obtain information about acyl chain order in the bilayer and Tt values for the SMs in these subfractions, the thermal behavior of liposomes prepared with them was assessed by determining the GP of Laurdan and the anisotropy of DPH as a function of temperature **(Fig. 1)**. The S+M SM subfraction presented a thermal profile similar to the one observed for total SM, namely high and low GP values at low and high temperatures, respectively, with an abrupt change of GP values between these two extremes. This change corresponds to a gel-liquid crystalline phase transition, the Tt calculated from the curves being 32.4°C for total SM and 36.6°C for S+M SM. The fact that the Tt was higher and the thermal transition was sharper for S+M SM than for total SM (Fig. 1A) was congruent with the species composition of each SM mixture, as total SM was more heterogeneous in molecular species composition than the S+M subfraction, which was relatively richer in 16:0 SM with a known Tt of ~42°C.

Similar experiments performed with the n-V SM and h-V SM groups of species showed that, even at the lowest temperature studied (5°C), the system was already above its Tt in both cases. Thus, although GP values gradually decreased as temperature increased, no lipid phase transition was observed. At all the tested temperatures, higher GP values were observed for the h-V SM group than for the n-V SM group. Interestingly, at higher temperatures (see, for example 45°C), Laurdan GP values were higher for these two groups of SM species than for total SM or for the S+M SM subfraction.

Using DPH as the fluorescent probe (Fig. 1B), gel to liquid-crytalline phase changes giving similar Tt values as with Laurdan were observed for total SM and S+M SM (32.6°C and 37.0°C, respectively). Again, the Tt for n-V SM and h-V SM fractions showed to be located below the range of temperatures tested, and *r* values were higher for h-V SM fraction than for n-V SM fraction within the studied range of temperatures. However, in contrast to what was observed with Laurdan, at higher temperatures (see, for example, 45°C), all groups of SM species showed rather similar DPH anisotropy values, indicating that they were all at a similarly fluid phase state.

Useful information about the membrane phase state within the bilayer in liposomes made of the SMs under study could be obtained by monitoring the environment of Laurdan, making use of the wavelength dependence of the GP spectra of the probe at various temperatures (**Fig. 2**). Gel phases are known to exhibit a wavelength-independent GP spectrum whereas liquid-crystalline phases display wavelength dependence for this parameter due to the dipolar relaxation process of Laurdan molecules (9, 21, 22). The explanation for this feature is that fluorophore-solvent interactions differ at the ground and the excited states of Laurdan because its dipole moment changes upon excitation and because the rate of reorientation of solvent molecules depends on the phase state of the membrane, being slower for gel and faster for liquid-crystalline phases (15). In liposomes such as those used in the present work, the main solvent dipoles are a few water molecules localized at the membrane-water interface. In the liquid-crystalline phase, the reorientation of these water molecules is possible and it

TABLE 2. Fatty acid composition (%) of the three groups of SM species used in this study

S+M SM		n-V SM		h-V SM	
16:0	46.0	n-28:4	52.8	h-28:4	37.8
18:0	31.3	n-28:5	2.7	h-28:5	2.1
24:0	5.2	n-30:5	25.5	h-30:5	44.7
24:1	6.2	n-32:5	5.5	h-32:5	5.4
Totals[a]	88.7		86.5		90.0

The SM groups of species were isolated from adult rat testis after the combination of TLC procedures described in Materials and Methods.

[a]The remainder percentage with respect to 100% was accounted for by fatty acids present in lower amounts than the depicted ones (see, for example, Fig. 3).

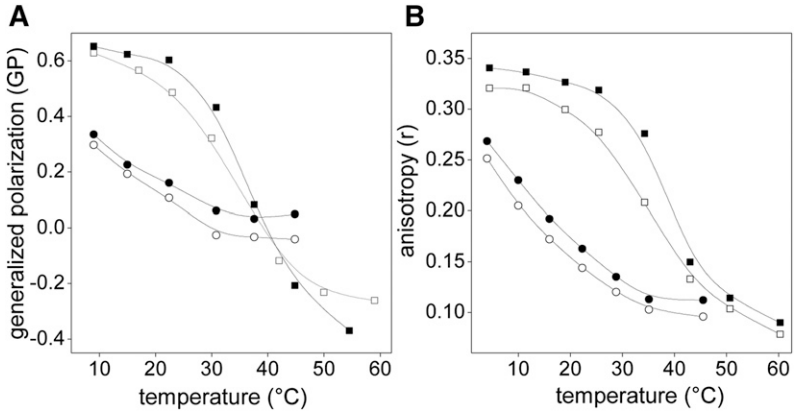

Fig. 1. A: Generalized polarization (GP) of Laurdan and (B) anisotropy of DPH (*r*) as a function of temperature in bilayers made of rat testis SM. Total SM (□) is compared with the three groups of SM species depicted in Table 2: S+M SM (■), n-V SM (○) and h-V SM (●). Data correspond to a representative measurement from a total of three or more independent experiments.

is of the same temporal order of magnitude as the fluorescence lifetime of Laurdan, so that the excited state dipole of Laurdan is able to align neighboring solvent dipoles. As a consequence, the exGP values decrease while emGP values increase with increasing emission wavelength.

The wavelength dependence of Laurdan GP in the three SM subfractions, S+M SM, n-V SM, and h-V SM, as a function of increasing temperature (3–38°C) is compared in Fig. 2. The S+M SM group showed two types of curves as a function of temperature (Fig. 2A). At low temperatures, exGP and emGP curves were quite independent of wavelength; as temperature increased, the curves started to display the wavelength dependence typical of liquid-crystalline phases, accompanied by low GP values. In the case of SM n-V and SM h-V (Fig. 2B and 2C, respectively), the curves evidenced a behavior highly dependent on the wavelength at all the temperatures tested, similar to that of S+M SM at high temperatures.

Considering that n-V SM and h-V SM subfractions were composed of different proportions of SM species, an n-6 tetraenoic fatty acid with 28 carbon atoms and an n-6 pentaenoic fatty acid with 30 carbon atoms predominating in the former and the latter, respectively (Table 2), the question arose as to whether the differences observed among these two subfractions in Fig. 1 were due to the presence of the hydroxyl group or to the presence of fatty acids with longer chains in the latter.

In order to gain information about the effects of the hydroxyl group, the hydrocarbon chain length, and the number of double bonds on the thermal behavior of the major individual species within each of these groups, individual molecular species were required. To this end, we developed the HPLC procedure described in Materials and Methods and applied it to the n-V SM and h-V SM subfractions to obtain sufficient amounts of n-28:4 SM, n-30:5 SM and n-32:5 SM on the one hand, and h-28:4 SM, h-30:5 SM and h-32:5 SM on the other (**Fig. 3**).

The thermal behavior of the major individual species thus obtained was studied with Laurdan and with DPH (**Fig. 4**) as fluorescent probes. As it occurred with the n-V SM or h-V SM subfractions, the GP versus temperature curves of four SM species, n-28:4 SM and h-28:4

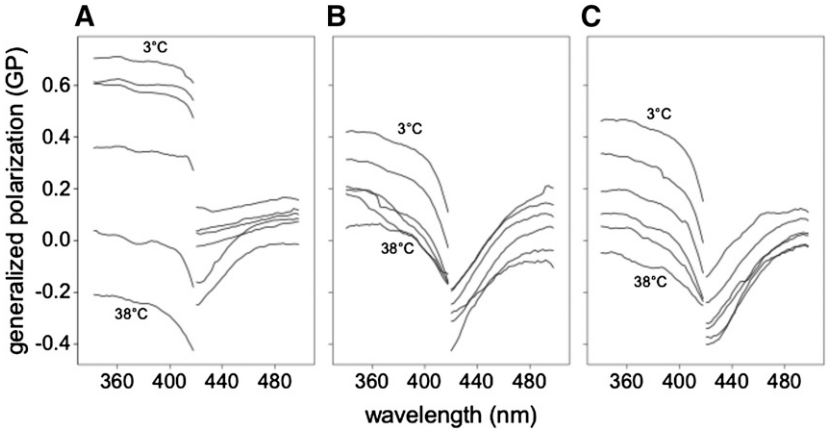

Fig. 2. Dependence of generalized polarization (GP) values of Laurdan on the wavelength when it is included in liposomes prepared with the three SM subfractions shown in Table 2: (A) S+M SM, (B) n-V SM, and (C) h-V SM. GP values were obtained as a function of excitation (320–415 nm) and emission (420–500 nm) wavelengths. Within each plot, the curves correspond to measurements carried out at different temperatures: 3°C, 9°C, 15°C, 22°C, 31°C, and 38°C, from top to bottom.

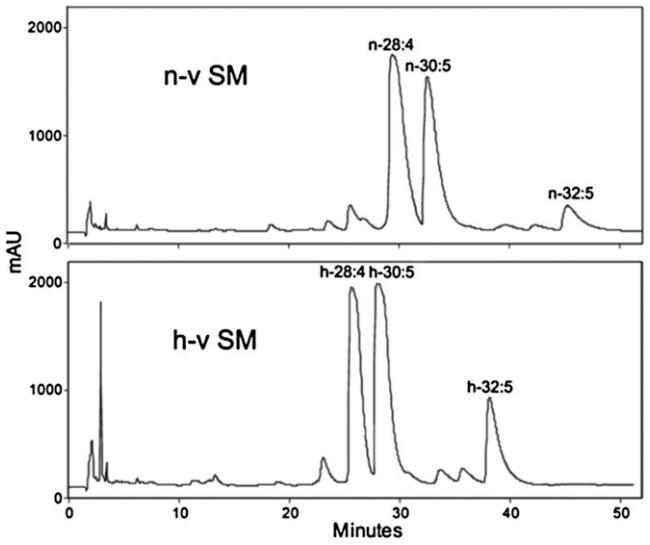

Fig. 3. HPLC separation of VLCPUFA-containing molecular species of rat testicular SM. Once separated by the combination of TLC procedures described in Materials and Methods, the n-V SM and h-V SM subfractions were subjected to HPLC to separate and collect the six major individual molecular species employed in the experiments shown in Fig. 4. Chromatography was performed using an octysilane (C8) column with a mobile phase of methanol:phosphate buffer flowing at 1 ml/min and a detector set at a wavelength of 203 nm.

SM, n-30:5 SM and h-30:5 SM, displayed a gradual decrease of their values without showing any inflection (Fig. 4A), an indication that their Tt values were below the surveyed range of temperatures. At all temperatures, GP values for two chain-matched SM species were higher when the species was 2-hydroxylated. Only in the case of n-32:5 and h-32:5 SM, the curves of GP of Laurdan (Fig. 4B) displayed an inflection from high to low GP values as a function of temperature that allowed to calculate a value of Tt for these two molecular species (21.7 and 22.8°C, respectively).

To ensure that the difference related to the hydroxyl group was statistically significant for the species displaying no Tt, namely those SMs containing 28 and 30 carbon atoms, we carried out an ANCOVA, which requires that each set of data in curve form can be fitted to a straight line. The Box Cox linearity transformation test applied to each data group indicated that they were best fitted by linear regression. In the case of n-28:4 SM versus h-28:4 SM, ANCOVA indicated that the two lines of GP as a function of temperature were not the same. Similar was the case for n-30:5 SM versus h-30:5 SM, their GP lines also differing. Thus, the 2-hydroxyl group must be credited for the larger GP values observed for each of the three h-V SM species than for their chain-matched n-V SM.

When the GP curves from the SM species under study were individually compared in order to evaluate the influence of the number of carbon atoms and double bonds (n-28:4 SM versus n-30:5 SM on the one hand and h-28:4 SM versus h-30:5 SM on the other), the results were not statistically different (same y-intercept and same slope; $P > 0.4$ in all cases) in spite of the two-carbon-atom difference between both species in each pair.

The alternative explanations for this similarity were either that the increase of two carbon atoms was compensated by the presence of an additional double bond in the fatty acid, or that Laurdan did not sense the difference between these SM species because of its relatively superficial position in the membrane.

To discern between these two possibilities, additional experiments comparing h-V 28:4 SM and h-30:5 SM were carried out using DPH (Fig. 4C), which localizes more deeply in the membrane. The tendency of the curves as a function of temperature was similar to the ones with Laurdan in the sense that low anisotropy values that gradually decreased

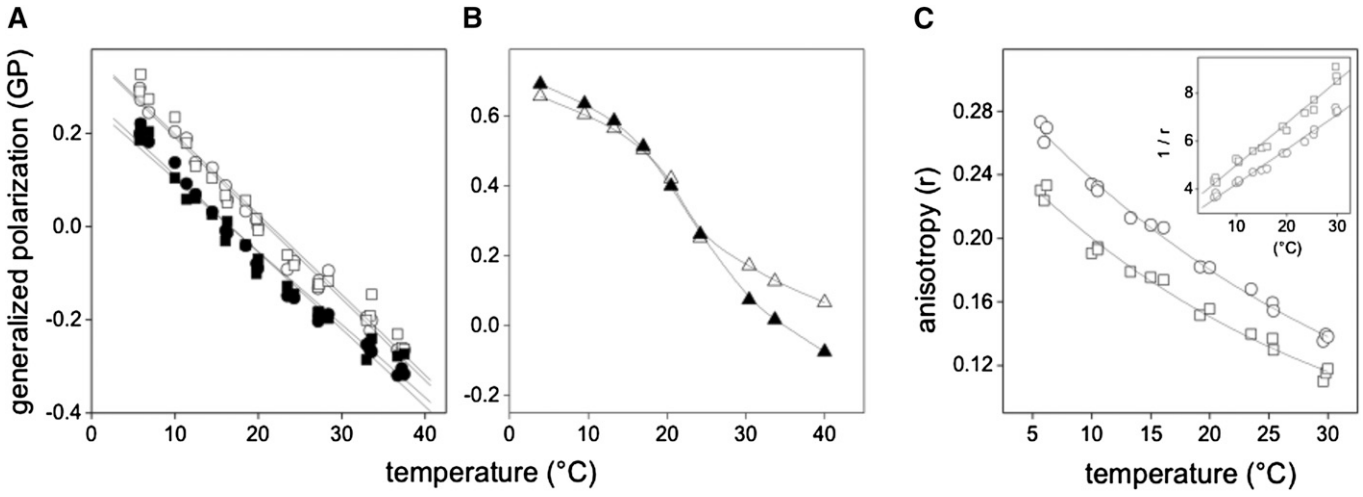

Fig. 4. Thermotropic behavior of individual VLCPUFA-containing molecular species of SM. A: Generalized polarization (GP) of Laurdan as a function of temperature in liposomes prepared with the major n-V SM and h-V SM species obtained by HPLC: 28:4 species (●, n-28:4 SM and ○, h-28:4 SM); 30:5 species (■, n-30:5 SM and □, h-30:5 SM). B: Similar measurements carried out with the two 32:5 species (▲, n-32:5 SM and △, h-32:5 SM). C: Anisotropy of DPH (r) as a function of temperature in liposomes prepared with h-28:4 SM (○) and h-30:5 SM (□). In the inset, the reciprocal of r values as a function of temperature are shown. All plots present the average from at least three independent experiments.

with increasing temperature showing no inflections were obtained. However, the use of DPH allowed the observation that the *r* curve of h-30:5 SM displayed significantly higher anisotropy values than that of h-28:4 SM. ANCOVA confirmed this observation. The Box Cox test suggested a reciprocal transformation of the data on the *y* axis (namely $1/r$) in order to achieve data linearity (Fig. 4C inset). The difference between the slopes of these lines for h-28:4 SM and h-30:5 SM was statistically significant. Thus, for this pair, the increase in one double bond caused a larger effect in decreasing *r* values, and hence membrane order, than the increase in two carbon atoms in increasing them.

These results indicate, on the one hand, that DPH was a better probe than Laurdan to sense perturbations in the core of the bilayer induced by both the number of carbon atoms and the number of double bonds. On the other, Laurdan displayed a higher sensitivity than DPH to distinguish differences between n-V and h-V SM species, this being consistent with the fact that in the latter case the 2-hydroxyl group of the SM fatty acid is more superficially located in the bilayer.

Comparing the effects of the three main characteristics that condition the whole biophysical behavior of the present SM species, namely fatty acyl chain length, number of double bonds and the presence or absence of the 2-hydroxyl group, the latter always had the predominant effect. The differences observed between n-V SM and h-SM subfractions (Figs. 1 and 2) could therefore be mostly attributed to the presence of the 2-hydroxyl group in the latter. Thus, we decided to use directly these subfractions to evaluate their interactions with other lipids and the possible impact of the presence of the 2-hydroxyl group in their acyl chain in these interactions.

Binary PC-SM systems

In order to evaluate the effect of VLCPUFA-containing SM on the thermal behavior of membrane GPL, liposomes containing diverse proportions of DMPC and n-V or h-V SM were prepared and the Tt of each sample was measured, again using the S+M SM subfraction as a reference (**Table 3**). The Tt of DMPC, ~24°C, increased as the proportion of the latter SMs increased. Conversely, it decreased as the proportion of n-V SM or h-V SM increased. Even as little as 15% of any of the above-mentioned SMs sufficed to change significantly the Tt of DMPC (Table 3 and supplementary Fig. I). In the case of n- or h-V SM, a proportion of 70% decreased the Tt to the extent that it fell at a value located somewhere below 5°C, the lowest temperature assayed in the present study. These disparities allow the inference that the presence of S+M SM increases, while that of n-V or h-V SM decreases, the lipid order in membranes at physiological temperatures.

Although both VLCPUFA-containing SMs decreased significantly the Tt of DMPC in a concentration-dependent manner, at higher concentrations it was possible to observe that the n-V SMs decreased it to a significantly larger extent than the h-V SMs (Table 3 and supplementary Fig. I) This was in agreement with the differences observed between both subfractions when they were alone in liposomes (Fig. 1).

Ternary PC-cholesterol-SM systems

Taking into account the remarkably different structural characteristics (chain length, degree of unsaturation, mismatch between hydrophobic chains) the n-V SM or h-V SM display with respect to the best studied saturated 14 to 24 carbon containing SM species (23), the hypothesis that their interactions with cholesterol also differ was evaluated. To test the likelihood that these SM would be resistant to promote cholesterol-SM domain formation, the protocol described by Xu and London (18) was used. Liposomes containing DOPC and/or the fluorescence quencher 10-SLPC, cholesterol, the different SM species, and DPH were prepared in the proportions indicated in the Materials and Methods section. The emission fluorescence of DPH was measured as a function of temperature in samples and their corresponding controls with no DPH, and the value of the *EF* was calculated.

To better understand the process of domain formation, the following considerations must be taken into account (18). On the one hand, DPH has no preference for lipid domains, localizing both in the disordered DOPC phase and in ordered cholesterol/SM domains. On the other hand, the nitroxide group of 10-SLPC disrupts the lipid packing of membranes in a manner similar to that of the double bond of an unsaturated lipid, thus displaying a phase behavior similar to that of DOPC. As a result, when a liposome preparation whose lipids are in a disordered state contains DPH and the fluorescence quencher 10-SLPC, the fluorescence detected is low, as is the calculated *EF* value. When cholesterol/SM-rich domains are formed, DPH remains localized in these domains but 10-SLPC is excluded, preferring the

TABLE 3. Differential effects of SM species on the Tt of DMPC

	Transition Temperature (°C)			
DMPC/SM	100/0	85/15	70/30	50/50
No SM	24.8 ± 0.4	—	—	—
S+M SM	—	28.1 ± 0.1[a]	30.9 ± 0.1[a]	33.5 ± 0.1[a]
n-V SM	—	21.9 ± 0.2[a]	19.0 ± 0.4[a]	15.7 ± 0.9[a]
h-V SM	—	21.9 ± 0.1[a]	20.9 ± 0.2[a,b]	18.0 ± 0.5[a,c]

The PC/SM ratios are expressed as mole %. Each transition temperature corresponds to mean values ± SD from at least three independent experiments.

[a]Statistically significant increases or decreases of Tt with respect to that of pure DMPC.

[b, c]Statistically significant differences between n-V SM and h-V SM species ([b]$P < 0.05$; [c]$P < 0.01$).

fluid lipid phase. This results in high DPH fluorescence emission revealed by high values of *EF*. As temperature increases the system evolves to disorder; when temperature exceeds the Tt of the SM, the domains disrupt, allowing DPH and 10-SLPC to take contact; the DPH fluorescence signal thereby decreases, and so does the *EF* value.

The *EF* values were obtained for n-V SM and h-V SM groups of species in comparison with 16:0 SM (**Fig. 5**). The latter SM showed the expected high and constant *EF* values as the temperature increased until it reached the Tt (42°C), with *EF* sharply decreasing thereafter. In contrast, n-V SM and h-V SM exhibited constant and considerably lower *EF* values in all the range of temperatures studied. Similar results to those shown in Fig. 5 were obtained when the proportion of cholesterol was increased from 15 to 30 mol % (data not shown). This confirms the conclusion that n-V SM and h-V SM have both a very low Tt. Because SM molecules must be below their Tt to be involved in the formation of SM/cholesterol-rich domains, it could be concluded that these SMs do not normally form such domains at physiological temperatures.

To confirm that n-V or h-V SM do not favor the formation of discrete lipid domains in the presence of cholesterol, direct observation using confocal microscopy in GUVs was performed. The latter, containing DOPC, cholesterol, and each of the SM species to be tested in a 1:1:1 molar ratio, were labeled with the fluorescence probe DiI, which has a strong preference for disordered lipid domains. In agreement with our spectrofluorimetric studies, the results (supplementary Fig. II) showed that SM/cholesterol-rich domain formation occurred when GUVs contained 16:0 SM and the temperature was below the Tt of this species. At comparable temperatures (20°C in supplementary Fig. II), GUVs containing n-V SM and h-V SM remained homogeneously fluorescent.

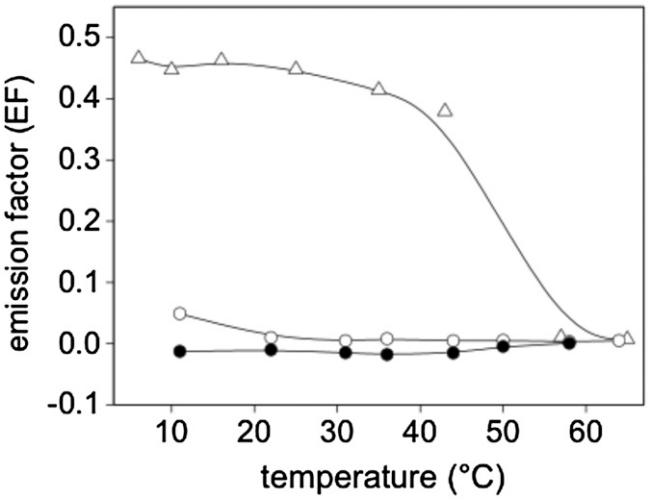

Fig. 5. Emission factor (*EF*) as a measure of domain formation by different SM species [16:0 SM (△), n-V SM (○) and h-V SM (●)] using the quenching of DPH fluorescence by 10-SLPC. *EF* corresponds to the difference between the fluorescence of DPH in samples containing each of the SM species and 10-SLPC (F_{SM}) normalized to that in samples containing each of the SM species and DOPC ($(F_0)_{SM}$) and the fluorescence of DPH in samples containing DOPC and 10-SLPC (F_{DOPC}) normalized to that in samples containing only DOPC ($(F_0)_{DOPC}$). Data are representative of at least three independent experiments.

DISCUSSION

The present study shows that the unique VLCPUFA-containing SM molecular species typical of rat spermatogenic cells and spermatozoa display biophysical properties and behavior that are closer to those of unsaturated fatty acid-containing GPL than to those of more common, abundant and ubiquitous long-chain saturated fatty acid-containing SM species. Thus, 16:0 SM, which in fact abounds in testicular cells other than germ cells, has a Tt of 42°C whereas the only Tts that could be determined for VLCPUFA-containing SM molecular species, corresponding to n-32:5 SM and h-32:5 SM, were in both cases close to 22°C. In liposomes of pure polar lipids, the Tt represents the temperature necessary to transform an ordered gel phase into a disordered liquid crystalline phase. Our results led to the conclusion that the n-V and h-V SM species of rat germ and sperm cells, irrespective of the presence or absence of the 2-hydroxyl group, share the common characteristic of having their very long acyl chains in the disordered state at physiological temperatures. The increase in Tt that could have resulted from their unusually long carbon chains was thus overridden by the effect that the large number of double bonds had in decreasing it.

The presence of the hydroxyl group located in the second carbon atom of the fatty acid in h-V SMs was key to increasing the GP values of h-V SM over those of n-V SMs at all temperatures tested, rather than the differences in the chain length and unsaturation between the SMs composing the n-V SM and h-V SM subfractions. This indicates that h-V SMs were, at all temperatures tested, in a relatively more ordered state than their n-V SM counterparts. The presence of the 2-hydroxyl group in h-V SMs apparently introduces an additional force attracting the molecules among themselves in the bilayers, namely that provided by intermolecular hydrogen bonding. This conclusion is supported by studies on the biophysical properties of glycosphingolipids, where the presence of a 2-hydroxylation in the acyl chain stabilizes their interactions and increases their melting temperatures (24, 25). This was also observed in SMs obtained by synthesis, as 2-hydroxylated SMs (h-16:0 SM and h-22:0 SM) were found to have gel-liquid transition temperatures 5–10°C higher than those of their non-hydroxylated counterparts (16:0 SM and 22:0 SM) (26).

The above-described differences due to the hydroxyl group were also observed when using DPH to measure anisotropy (Fig. 1). Laurdan is mostly positioned at the hydrophilic-hydrophobic interface (27) with its lauric acid tail interdigitating in the acyl chain region closer to the membrane surface, while DPH tends to localize more deeply, toward the membrane hydrophobic core (28). Thus, while the latter typically senses variations in deeper regions of bilayer membranes, Laurdan discriminates better lipid features in the bilayer region closer to the carboxyl groups.

The different location of the probes gave complementary information. The fact that at high temperatures (e. g., 45°C) the *r* values were similarly low for the fractions enriched in SM with saturated fatty acids, n-V SMs and h-V SMs (Fig. 1) indicates that, at the hydrophobic core of the membrane, there is a similarly high degree of disorder at this temperature in the three cases. Considering that the n-V SM and h-V SM fatty acids belong to the n-6 series, the superficially located Laurdan would make contact with the saturated segment of the acyl chain that goes from the carboxyl group to the first of the series of double bonds in (n- or h-) 32:5, 28:4 and 30:5 SMs (which are 14, 13, and 12 carbon atoms, respectively), rather than with the more deeply localized long segments these fatty acids have toward their methyl end (i.e., the series of carbons involved in the 4 or 5 methylene-interrupted *cis* double bonds). At these temperatures, it may be expected that all fatty acid segments in the bilayer are highly mobile in all SMs, and also that the SM molecules themselves are free to undergo unrestricted lateral mobility in the plane of the membrane. Although this may be the case for those SM species containing straight, fully saturated acyl chains, the unusually long and highly unsaturated portion the n-V and h-V SMs have in relatively deep regions of the SM bilayers may be bulky and kinky enough to impose a certain degree of restriction to the free lateral mobility of these SM molecules. This could help explain the intriguing observation that, at high temperatures, the GP values with Laurdan were higher for n-V SM and h-V SM than for total SM or for the S+M subfraction.

The isolation of individual SM molecular species containing VLCPUFA allowed us to conclude that differences due to chain length or degree of unsaturation of the SM species under study would not be discriminated by Laurdan, as shown for SMs with n-28:4 to n-32:5 on the one hand, and h-28:4 to h-32:5 on the other in Fig. 4. This may be attributed in part to the small differences there are among these fatty acids in the saturated segment that goes from the carboxyl group to the first of the series of methylene interrupted *cis* double bonds (13 carbons in 28:4, 14 carbons in 32:5). In contrast, by comparing the behavior of h-28:4 SM and h-30:5 SM, DPH allowed us to observe that the presence of an extra double bond in the fatty acid exerts a stronger effect in decreasing membrane order than the increase in two carbon atoms has in increasing it.

The fact that except for the longest n-32:5 SM and h-32:5 SM, the Tt could not be estimated for the rest of the n- or h- SM species tested in our study indicates that their phase transition must occur at lower (probably below-zero) temperatures. Taking into account experimental data from Marsh Laboratory (29), we observed that the Tt of a homologous series of 16 to 24 carbon atom SM saturated species may be related to the number of carbon atoms in the fatty acid chain (N) by the following expression:

$$\mathrm{Tt}(^{\circ}\mathrm{C}) = 27.17 + 0.91 \times \mathrm{N} \qquad \textit{Eq. 5}$$

Thus, an SM molecule having a saturated 32 carbon atom fatty acyl chain would have a Tt of 56.3°C, i.e., 30°C higher than the experimental value of ~21°C we observed for the Tt of n-32:5 SM. This underscores the large reducing impact that the introduction of the double bonds has in determining the Tt of 32:5 SM.

Using the above expression, it may be estimated that the increase in eight carbon atoms from 16:0 SM to 24:0 SM increases Tt by just 7.3°C, each increase in two carbon atoms in the 16:0-to-24:0 series contributing a stepwise increase of just 1.82°C. The higher than 20°C difference in Tt due to the 2-carbon atoms decrease between n- or h- 32:5 SM (around 22°C) and n- or h- 30:5 SM (below zero) was, in contrast, considerable. It was comparable to the difference in Tt produced by the 6-carbon atom decrease between 24:1n-9 SM, with a Tt of 24°C (30), and 18:1n-9 SM, with a Tt falling at a below-zero temperature (31).

n-V SM and h-V SM interactions with lipids

The interactions between SM and DMPC showed opposite effects for liposomes containing n-V or h-V SMs on the one hand and S+M SMs on the other, the former decreasing and the latter increasing the Tt in a binary PC:SM system with respect to the Tt of pure DMPC (23°C). Interestingly, whereas increasing proportions of an unsaturated PC added to a model membrane containing a saturated SM such as 16:0 SM progressively decreased the Tt of the latter (32), in the present study, the addition of increasing proportions of highly unsaturated SMs decreased the Tt of a saturated glycerophospholipid such as DMPC (Table 3). Thus, in this case, n-V and h-V SMs played the role of the "fluidizing" lipid of the system, contrary to the general consensus that SMs have a lipid mobility-restraining effect in bilayers.

Both the n-V and h-V SM subfractions induced a decreasing effect on the Tt of DMPC, and the magnitude of this decrease became significantly different as their proportion increased. In addition, DMPC Tt was decreased to a larger extent by n-V SM than by h-V SM, in line with the differences in GP values observed when each of these two groups was the only component of the system. This difference could allow the extrapolation that there is a higher degree of disorder in rat pachytene spermatocyte membranes, which contain virtually only n-V SMs, than in spermatids, which become increasingly rich in h-V SMs as they differentiate to become spermatozoa (7). However, although each type of SM predominates in each type of cell, other lipids like the abundant glycerophospholipids that contain 20 and 22 carbon tetraenoic and pentaenoic PUFA may contribute to create, and probably prevail in determining, a mainly disordered state.

The experiments with ternary DOPC:cholesterol:SM systems, one evaluating the quenching of DPH fluorescence by 10-SLPC and the other one following the distribution of the fluorescent probe DiI in GUVs, proved correct our initial presumption that the n-V and h-V SMs would be "resistant" to segregate into cholesterol/SM-rich domains when they are in coexistence with cholesterol, at least at temperatures at which most other SM species typically do (see for example (23) and references therein).

The reported tendency of 14 to 24 carbon saturated fatty acid-containing SM to segregate into lateral-ordered domains with cholesterol is favored by the high SM Tt values and by the low miscibility of the disordered lipids of the system (in this case DOPC) in these relatively compact domains. The fact that this phenomenon was not observed when n-V or h-V SM subfractions were present is consistent with these SMs having low Tt values, and suggests that at physiological temperatures, they may have a higher propensity to interact with lipids in a disordered state than with cholesterol.

In membranes where SM species with VLCPUFA and saturated fatty acids coexist, the liquid-ordered domains are likely to include most of the latter, whereas the more disordered membrane areas, comparatively GPL-richer and cholesterol-poorer, may contain most of the former. This possibility was supported by results in bull spermatozoa, which contain a high proportion of SM (nearly 20% of total sperm lipid phosphorus) exceedingly rich in n-VLCPUFA (~70% of SM fatty acids) (33). After exposing isolated heads from these gametes to Triton X-100 at 4°C, virtually all of these n-V SM species were extracted in the detergent-soluble membrane fraction, while the lipid remaining in the detergent-insoluble fraction was rich in the relatively minor saturated SMs, mostly 16:0 SM.

Despite its high degree of unsaturation, the segment of 14 carbon atoms n-32:5n-6 SM has, in the saturated portion of its fatty acid, allowed this species to undergo a measurable temperature transition at around 21°C. Considering that SM species containing n-32:6n-3, 34:6n-3, 32:5n-3, and 34:5n-3 abound in bull and ram sperm (33), for which this segment is 14, 16, 17 and 19 carbon atoms long, respectively, it is not unlikely that some of the latter may also undergo transitions in the 5-37°C range. This possibility may have practical implications for sperm fertility. Apparently, the abundant SMs of spermatozoa are naturally intended not to undergo thermotropic transitions and/or interactions with cholesterol as long as sperm are in their physiological environments at the temperature of male or female reproductive tracts. However, these transitions and/or interactions may occur when male gametes are for some time far from this temperature. This may be of concern at the chilly, and especially at the sub-zero, temperatures at which these gametes are usually preserved, whether in the research laboratory or with in vitro fertilization purposes.

The fluid, highly disordered lipid milieu normally provided by VLCPUFA-containing SM species to sperm membranes may be required for specific sperm proteins to be in their optimal functional state at bodily temperatures. Thus, inopportune lateral separation of these SMs from the immediate microenvironment of membrane proteins as temperature decreases, inducing them to coalesce into ordered SM-rich domains, or to become sequestered into SM/cholesterol-rich lateral domains, may cause protein dysfunctions that may range from irreversible protein denaturation and functionality loss to undesirable protein activation. This possibility is worth exploring among the factors leading to the inevitable decrease in fertility that cryopreserved gametes are known to undergo in comparison with freshly isolated counterparts rapidly manipulated at near physiological temperatures.

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