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Sphingomyelins of Rat Liver: Biliary Enrichment with Molecular Species Containing 16:0 Fatty Acids as Compared to Canalicular-Enriched Plasma Membranes

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Abstract. We harvested canalicular-enriched plasma membranes of hepatocytes and collected fistula bile from male rats and isolated the sphingomyelins. Following sphingomyelinase hydrolysis, we identified the sphingomyelin molecular species on the basis of their benzoylated ceramide derivatives employing high performance liquid chromatography. Sphingomyelin constitutes ⩽3% of total biliary phospholipids (which are mostly sn-1 16:0 long-chain phosphatidylcholines) and approximately 30% of canalicular-enriched membranes. In both cases, the principal molecular species were composed of 16:0, 18:0, 20:0, 22:0, 23:0, 24:0, 24:1 and 24:2 fatty acid classes. However, the 16:0 fatty acid species was enriched in biliary sphingomyelin to a significantly greater degree than in sphingomyelins of canalicular-enriched plasma membranes (46% *vs.* 25% of total). We argue a physical-chemical case for laterally separated domains of very long chain sphingomyelins on the exoplasmic leaflet of the canalicular membrane. We bolster our hypothesis by the likelihood that the least hydrophobic, e.g., 16:0 sphingomyelin molecular species, are miscible with biliary phosphatidylcholines, and are secreted into bile. Laterally separated domains of very long chain sphingomyelins on the exoplasmic leaflet of the canalicular membrane could provide a means of sequestering cholesterol molecules prior to secretion into bile.

Key words: Liver — Canalicular plasma membrane — Membrane-enriched subfractions — Bile — Sphingomyelin — Fatty acids

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Introduction

The canalicular membrane, through which biliary lipids are secreted, comprises 10–15% of the total plasma membrane of the hepatocyte. It is functionally and biophysically distinct, containing a host of biliary lipid export pumps (Müller & Jansen, 1997; Trauner, 1997) as well as higher levels of cholesterol and SM than the basolateral plasma membrane (Kremmer, Wisher & Evans, 1976; Schachter, 1988). Sphingomyelin (SM) and phosphatidylcholine (PC) are the major structural phospholipids on the outer leaflet of the hepatocyte canalicular plasma membrane and are reported to occur in a ratio as high as 0.7 (Higgins & Evans, 1978; Schachter, 1988). Most recent research has focused on SM's hydrolytic products, which have important signaling effects, particularly in the gastrointestinal tract, regulating cell proliferation, differentiation and apoptosis (Merrill & Jones, 1990; Kolesnick, 1991; Hannun & Linardic, 1993; Spiegel & Merrill, 1996). In bile of both humans and laboratory animals, PC is the predominant (>95%) phospholipid class, whereas SM comprises only 1–3% of total phospholipids (Alvaro et al., 1986; Barnwell, Tuchweber, & Yousef, 1987). During bile formation, PC molecules are eluted as vesicles from the outer leaflet of the canalicular plasma membrane (Crawford et al., 1995) and it is believed that they are dissolved quickly by micellar bile salts to form mixed micelles (Cohen & Carey, 1990). Since natural SM has a higher affinity than other phospholipids for cholesterol, the tissue levels of SM correlate positively with the amount of cholesterol in membranes (Demel et al., 1977; Lund-Katz et al., 1988; Slotte, 1995). In the hepatocyte, SM and the majority of cholesterol molecules are present in the plasma membrane (Lange et al., 1989). Hydrolysis of plasma membrane SM by exogenous sphingomyelinase, as evidenced by studies with cultured fibroblasts and intestinal

absorptive cells, alters cellular cholesterol storage, esterification, biosynthesis and secretion (Slotte & Bierman, 1988; Gupta & Rudney, 1991; Chen et al., 1992). In previous work, we have shown in vitro, employing a model system, that the high affinity of natural SM (buttermilk, brain and egg yolk SM) for cholesterol is lost in the presence of submicellar bile salt concentrations (van Erpecum & Carey, 1997). Canalicular SM, with its putative role in cholesterol sequestration, may influence bile formation and biliary cholesterol secretion. Therefore, the molecular species of SM in the canalicular plasma membrane and bile should provide clues for understanding interactions between SM with other membrane lipids and cholesterol. We have now defined the SM molecular species of canalicular-enriched plasma membranes (cLPM) and hepatic bile of male Sprague-Dawley rats. We found a bimodal distribution of SM fatty acid species both in cLPM and bile, which is consistent with SM molecular species in other organs. We found marked differences in the relative distributions of long- and very long-chain fatty acids between the cLPM and bile, suggesting preferential biliary secretion of 16:0 SM molecular species. These results predict not only partial miscibility of canalicular SM species, but the possibility of phase-separated SM domains on the canalicular membrane that may function as reservoirs for biliary cholesterol secretion.

Materials and Methods

Materials

Sucrose, benzoic anhydride, dimethylaminopyridine, glucose-6-phosphate, phenylmethyl-sulfonyl-fluoride (PMSF), neutral sphingomyelinase from Staphylococcus aureus and a kit for leucine aminopeptidase assay were obtained from Sigma Chemical, St. Louis, MO. Pentobarbital was purchased from Abbott laboratories, North Chicago, IL. Egg yolk SM, egg yolk PC and lysoPC (Avanti Polar Lipids, Alabaster, AL) all purified by preparative high performance liquid chromatography (HPLC), yielded single spots on thin layer chromatography (TLC) employing chloroform/methanol/water (65:25:4 v/v/v), with application of 200 μg lipid. Aluminum-backed silica G/UV plates (Whatman, Maidstone, Kent, England) were employed for high performance TLC. For normal phase HPLC, a 5 μm LiChrospher Si-100 (25 cm × 4 mm) column (Merck, Darmstadt, FRG) was used and for reversed phase HPLC, a 3 μm particle size Spherisorb ODS (25 cm × 2 mm) column (Metachem Technologies, Torrance, CA) was employed. All solvents were HPLC grade (Fisher Chemical Scientific, Fair Lawn, NJ) and mobile phases were filtered prior to use through a 0.5 μm Teflon FH filter (Millipore, Bedford, MA). Pyrex brand glassware was alkali-alcohol washed for 24 hr (EtOH-2M KOH 1:1, v/v), and then immersed for 24 hr in acid (1 M HNO_3) followed by thorough rinsing with purified water prior to drying. Water was filtered, ion-exchanged, and glass distilled (Corning Glass Works, Corning, NY).

Methods

Isolation of Canalicular Membrane-Enriched Subfractions from Rat Liver

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in accordance with the guidelines of the committee on animals of Harvard Medical School and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS publication, No. (NIH) 85-23, Revised 1985). Adult animals weighing between 200 and 250 g were supplied with regular chow and water *ad libitum.* Livers were harvested following decapitation in the early morning, circa 8:00 a.m. Liver plasma membranes were isolated from 6 to 8 rats at 4°C by sucrose density ultracentrifugation according to the method of Meier and colleagues (Meier et al., 1984) with minor modifications. In brief, rat livers were homogenized in 1 mM $NaHCO_3$ buffer with 0.1 mM PMSF (pH 7.4). After centrifugation for 90 min at 90,000 × *g*, plasma membrane fractions were recovered from the 36.5–44% sucrose interface. Plasma membranes were homogenized in 8.1% sucrose and recentrifuged at 200,000 × *g* for 3 hr followed by recovery of canalicular-enriched liver plasma membranes (cLPM's) from the 8.1–31% sucrose interface. Membrane subfractions were washed in 8.1% sucrose and centrifuged for 1 hr at 120,000 × *g*. Pellets were resuspended and revesiculated through a 25 gauge needle in 1 mM $NaHCO_3$ and stored in −70°C. For class and molecular species analysis, lipids of cLPM fractions were extracted by the method of Folch, Lees & Sloane Stanley (1957). Leucine aminopeptidase showed a 20-fold enrichment in the cLPM fractions whereas glucose-6-phosphatase revealed 10–15% microsomal contamination of the cLPM, which is in accordance with data of Meier et al. (1984).

Sampling of Rat Hepatic Bile

Adult male Sprague-Dawley rats were anesthetized between 8:00 and 9:00 a.m. with pentobarbital (50 mg/kg body weight), and body temperature was maintained at 37°C with a heating lamp. Through a midline abdominal incision, a PE-10 catheter (Becton Dickinson, Parsippany, NJ) was introduced into the common bile duct and bile samples were collected in 30 min intervals for 2 hr followed by immediate extraction of lipids (Folch, Lees & Sloane Stanley, 1957). After the last bile collection, rats were sacrificed with an overdose of pentobarbital.

Phospholipid Class Separation

A cLPM sample containing 1 μg protein, together with 300 μl hepatic bile were used for phospholipid class separations by HPLC (Patton, Fasulo & Robins, 1990*a*). After partitioning lipid extracts from cLPM and hepatic bile with 0.2 volumes of 0.15 M NaCl (Folch et al., 1957), the chloroform phase was harvested via a separatory funnel and dried under a stream of N_2. The dried samples were redissolved in 100 μl of the mobile phase and applied to the LiChrospher Si-100 column for normal phase HPLC. Separation of PC and SM from the other phospholipids and neutral lipids was achieved using acetonitrile/methanol/water (90: 55: 15, v/v/v) with a flow rate of 0.5 ml/min and absorbance monitored at 205 nm (Jungalwala, Evans & McCluer, 1976). The PC and SM peaks were collected separately, dried under N_2 and stored in $CHCl_3$/MeOH (2:1, v/v). Peaks of interest were analyzed by high performance TLC in chloroform/methanol/water (65: 25: 4, v/v/v) using 20 μg PC, SM and lysoPC, all from egg yolk, as standards.

Sphingomyelinase Hydrolysis and Benzoylation

HPLC peaks containing SM were hydrolyzed with 0.5 units of neutral sphingomyelinase (Staphylococcus aureus) in 0.5 ml buffer (50 mM Tris, 1 mM $MgCl_2$, pH 7.4) according to the method of Patton, Fasulo & Robins (1990*b*) and, the resulting ceramides were subsequently benozylated by the method of Blank et al. (1984). The benzoyl ceramide derivatives were purified by HPLC using a LiChrospher Si-100 column with a hexane/tetrahydrofuran/acetic acid (500: 35: 0.1, v/v/v) mobile phase at a flow rate of 1 ml/min and detected at 230 nm as described (Ullman & McCluer, 1977).

Molecular Species Separation

The benzoylated ceramides from the SMs of cLPM were applied to a 3 μm particle size Spherisorb ODS (25 cm × 2 mm) column for reverse phase HPLC. The benzoylated ceramides obtained from SMs of hepatic bile were added to two 5 μm particle size Ultrasphere ODS (25 cm × 2 mm) columns connected in series. Molecular species separation of benzoylated ceramides from membrane fractions and bile, was carried out with acetonitrile/methanol (1:1, v/v) at a flow rate of 0.3 ml/min (Patton & Robins, 1987). Based upon their HPLC retention times, the carbon numbers of the fatty acids were identified according to Kadowaki, Evans and McCluer (1984) with confirmation of molecular species identification by mass spectrometry (Patton, Fasulo & Robins, 1990*b*).

Mass Spectrometry of Benzoylated Ceramides

Identification of fatty acid molecular species was confirmed by Matrix Assisted Laser Desorption Ionisation (MALDI) Time-of-Flight (TOF) mass spectrometry (Karas et al., 1987; Hillenkamp & Karas, 1991) of the benozylated ceramides using a PerSeptive Biosystems Voyager-DE-RP, MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with delayed extraction (Vestal, Juhasz & Martin, 1995). A 337 nm UV Nitrogen laser producing 3 ns pulses was employed and the mass spectra were obtained in the linear and reflectron modes. Samples were prepared by mixing 10 μl of a 50% acetonitrile/water solution of the benzoyl ceramide derivatives with 20 μl of a solution of 3 mg dihydroxybenzoic acid in 12.5 mL water (containing 0.1% trifluoroacetic acid). One μl of the solution was loaded on a gold-sample plate, the solvent was removed in warm air and the sample transferred to the vacuum of the mass spectrometer for analysis.

Statistics

Data are expressed as means ± SD employing a commercial statistical software program (Sigmastat, supplied by Jandel, San Rafael, CA). A two-tailed *P*-value <0.05 was considered significant.

ABBREVIATIONS

cLPM, canalicular enriched liver plasma membranes; SM, sphingomyelin; PC, phosphatidylcholine; HPLC, high performance liquid chromatography.

Results

Figure 1 shows a representative chromatogram by normal phase HPLC of phospholipid classes in lipids extracted from cLPM. There is baseline separation of both PC and SM from the other phospho- and neutral lipids, with a clear distinction between peak heights of PC and SM. By high performance TLC, the separately collected PC peak showed a single spot, whereas the SM peak consisted of two spots migrating in close proximity to each other (*data not shown*). Extracted biliary phospholipids revealed similar chromatograms by normal phase HPLC, except that the PC peak was appreciably enhanced.

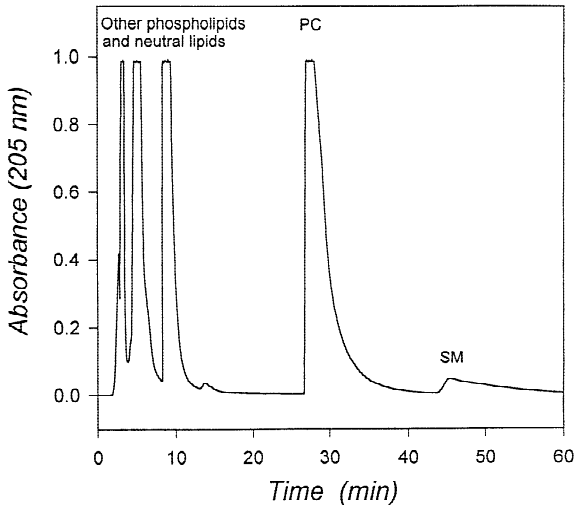

Fig. 1. HPLC separation of major classes of phospholipids of canalicular membrane-enriched subfractions. Chromatography was performed with a LiChrospher Si-100 column, a mobile phase of acetonitrile-methanol-water (90: 55: 15, v/v/v), a flow rate of 0.5 ml/min and a wavelength of 205 nm. A portion equal to 1 μg protein was dried under N_2, redissolved in the mobile phase and applied on the column. The PC peaks were collected between ~26 and ~43 min and the SM peaks between ~43 and ~60 min.

Figure 2 demonstrates purification and isolation by normal phase HPLC of benzoylated ceramides from the SMs of cLPM, showing that they elute much later than a number of benzoylated impurities. The asymmetrical bifid peaks collected between 15 and 20 min represent the benzoylated ceramide derivatives, which were then applied to a reverse phase HPLC column to separate the individual molecular species.

Figure 3 shows an HPLC chromatogram of the benzoylated ceramides representing the molecular species of SM in cLPM. Peaks were identified according to Patton et al. (1990*b*), with a minor difference in that we used a mobile phase containing acetonitrile to separate 24:1 from 22:0 benzoyl ceramide peaks. The abbreviations (Fig. 3) used to label molecular species are those of Breimer, Karlsson & Samuelsson (1974). For example, in the notation d18:1–16:0, the d18:1 represents the long chain sphingosine base (1,3 dihydroxy-2-aminooctadecene) and 16:0 represents the fatty acid (palmitic acid).

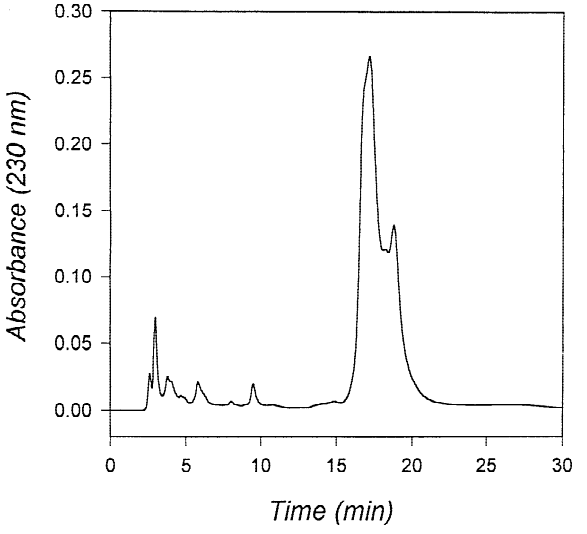

Fig. 2. HPLC purification of benzoylated ceramides of canalicular membrane-enriched subfractions. The benzoyl derivatives were isolated using a LiChrospher Si-100 column with hexane-tetrahydrofuran-acetic acid (50: 35: 0.1, v/v/v) mobile phase at a flow rate of 1 ml/min and detected at a wavelength of 230 nm. Benzoylated ceramides were collected between ~15 and ~21.5 min.

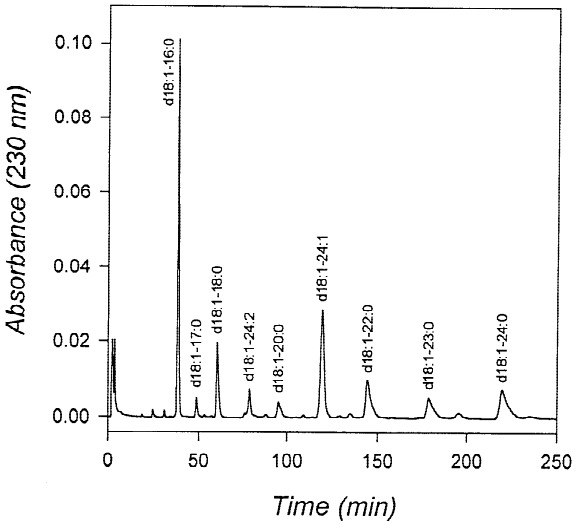

Fig. 3. HPLC separation of benzoylated ceramides of canalicular membrane-enriched subfractions. Chromatography was performed with a Spherisorb ODS 2 column, a mobile phase of methanol-acetonitrile (1:1, v/v), a flow rate of 0.3 ml/min and a wavelength of 230 nm. The molecular species were identified according to Patton et al. (1990) and confirmed by MALDI-TOF mass spectrometry.

Each assignment was confirmed by MALDI-TOF mass spectrometry. Figure 3 shows that SMs of cLPM contained mainly 16:0, 18:0 fatty acids and a series of fatty acids longer than 20:0. The chromatograms of benzoylated ceramides derived from the molecular species of SMs in bile are similar to cLPM, but marked differences

Table. Percent distribution of the SM molecular species of canalicular enriched hepatocyte membranes and bile from male rats

Fatty acid	cLPM (n = 5)	Hepatic bile (n = 6)
16:0	24.6 ± 4.0	45.8 ± 7.5*
18:0	8.0 ± 1.2	3.9 ± 0.8*
24:2	4.2 ± 0.8	5.4 ± 0.9
20:0	3.4 ± 0.2	1.5 ± 0.3*
24:1	22.1 ± 0.4	18.7 ± 2.7*
22:0	11.8 ± 0.8	8.3 ± 1.7*
23:0	9.0 ± 1.3	5.6 ± 1.0*
24:0	16.9 ± 3.7	10.9 ± 1.7*
Mean % fatty acids with >20 C-atoms	62	53
Mean % saturated fatty acids	74	73

The fatty acids, amide-linked to sphingosine (d18:1), are listed from top to bottom in order of increasing hydrophobicity. Results are given as means ± SD with the number of experiments in parentheses next to the column heading. Abbreviations: cLPM, canalicular enriched liver plasma membrane. Fatty acid nomenclature: number to left of colon represents number of carbon atoms in chain; number to right represents number of cis double bonds.
* $P < 0.05$; hepatic bile compared with cLPM.

in peak heights were noted, suggesting that the proportions of long to very long chain fatty acid were different.

The Table lists the percent distribution of the SM molecular species of cLPM and bile given in descending rank order of increasing hydrophobicity based on the elution sequence of the benzoylated ceramides (Fig. 3). The principal SM molecular species of cLPM and bile are 16:0, 18:0, 20:0, 22:0, 23:0, 24:0, 24:1 and 24:2 fatty acids, amide linked to sphingosine (d18:1). Of note is that hepatic bile is significantly ($P < 0.05$) more enriched in d18:1–16:0 (46%) SM, compared with the level (25%) in cLPM and the other (C18:0, C20:0, 24:1, 22:0, 23:0 and 24:0) SM species are reciprocally depleted. Although the mean percent distribution of saturated fatty acids remain similar, the relative amounts of very long chain SM's are highest in cLPM and least in bile. For purposes of illustration, Fig. 4 shows the fatty acid distributions of SM (Table) of SM's of cLPM and bile in order of increasing hydrophobicity. This affords visualization of the reciprocal distribution between the long- and very long-chain fatty acids in SMs of cLPM and bile.

Discussion

The lipid composition and molecular species distribution of individual lipid classes in the hepatocyte plasma membranes has been the subject of several studies (van Hoeven et al., 1975; Wisher & Evans, 1975; Kremmer et al., 1976; Schachter, 1988). Since all lipids entering bile must flow through cLPM, and since biliary PC is secreted by a vesiculation mechanism (Crawford et al.,

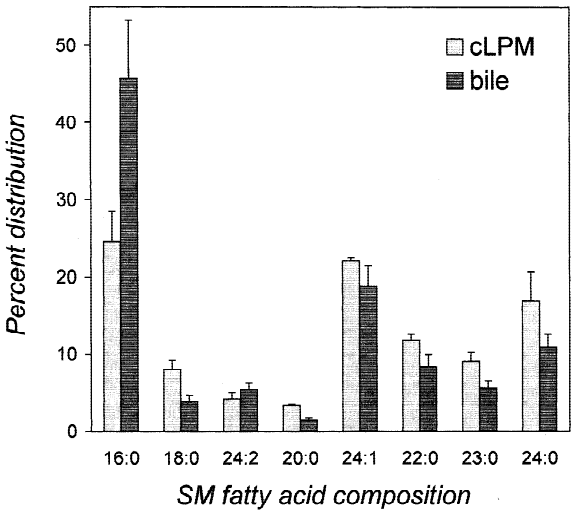

Fig. 4. Fatty acid compositions of SM's of canalicular membrane-enriched subfractions and in bile. The fatty acids, amide-linked to sphingosine (d18;1), are shown from left to right in order of increasing hydrophobicity. Results are given as means ± SD.

1995) we have addressed the molecular species of SM, the other major lipid on the exoplasmic face of the canalicular membrane of hepatocytes to determine whether the individual molecular species are the same in cLPM and in bile. Our results employing male rats are consistent with the typical chemical composition of mammalian SM, which contains principally saturated acyl chains, as was evidenced by the normal phase HPLC separation in our study (*see* Fig. 1) and the low response factor of SM at 205 nm, which detects double bonds. Further, the SM peak gave a dual spot by TLC (*see* results) and a dual peak by HPLC (Fig. 2), which suggested the presence of a bimodal distribution of long- and very long-acyl (>20 C-atoms) chains in SM as verified by HPLC analysis of the benzoylated ceramides (Fig. 3). As displayed in the Table and Fig. 4, the major long-chain fatty acids were 24:1 and 24:0, which accounted for the prominent early first part of the dual peak, whereas 16:0 fatty acids constitute most of the second part of the dual peak (Fig. 2). These findings are consistent with previously published data on SM fatty acid distribution of total plasma membranes of male and female rat hepatocytes (van Hoeven et al., 1975). Since no gender difference was noted in this earlier work, it was reasonable to carry out our study on male rats. The most commonly occurring sphingoid base in mammalian parenchymal cells, including liver, is sphingosine (4-sphingenine), which constitutes >90% of total bases in mammals (Samuelsson & Samuelsson, 1969; Hirvisalo & Renkonen, 1970), therefore it was unnecessary and indeed beyond the scope of the present work, to further address this issue.

In uniformity with all other studies in laboratory animals or humans, there are only small amounts of SM in rat biles (Alvaro et al., 1986). It is reasonable to assume therefore, that the small levels of biliary SM in animals biles (and in humans) are secreted at the level of cLPM, as is biliary PC, which is eluted from the outer hemi-membrane leaflet of the cLPM (Crawford et al., 1995) under the influence of a ''flippase'' (Smit et al., 1993) that promotes PCs transmembrane translocation. Therefore, the SM fatty acid distribution in bile may reflect those SM molecular species that are less hydrophobic and miscible with PC on the exoplasmic leaflet of the canalicular membrane.

Because the cLPM is believed to contain only traces of glycosphingolipid, including gangliosides, which have similar ceramide ''back-bones'' as in SM (Keenan, Huang & Morrè, 1972; Dnistrian et al., 1977; Matyas & Morrè, 1987), the degree of stiffness or molecular order of the cLPM may be attributed principally to SM and cholesterol molecules. Indeed, this appears to be aided substantially by the high cholesterol to phospholipid ratio of the cLPM (Schacther, 1988). The PC molecular species of the cLPM is mainly sn-1 16:0, and this is reflected in bile (van Hoeven et al., 1975; Alvaro et al., 1986; Barnwell et al., 1987). Both in monolayers and in bulk systems, extensive phase equilibria experiments (Shinitzky & Barenholz, 1974; Demel et al., 1977; Estep et al., 1980) suggest that sn-1 16:0 PCs and SMs with greater than C-20 chains will be hydrophobically mismatched and immiscible, and should form laterally separated domains of SM and PC in cLPM. As inferred from the current work, 16:0 SM may very well be miscible with sn-1 16:0 PC, the typical biliary subspecies, since the 16:0 SM molecular species is preferably concentrated in bile (Fig. 4, Table). Although cholesterol has no special preference for SM compared with PC when the fatty acid chain lengths are identical (Lange, D'Alessandro & Small, 1979), it is likely that cholesterol will phase separate into microdomains of naturally occurring SM containing >C20 fatty acids on the outer face of the cLPM (Slotte, 1995; Demel et al., 1977). Indeed, cholesterol may augment lateral phase separation of the longer chain species (Untrach & Shipley, 1977; Lentz, Hoechli & Barenholz, 1981), forcing more 16:0 SM's to mix with the natural PCs of the cLPM. Presumably, after being ''flipped'' by the mdr-2 encoded P-glycoprotein transmembrane transporter (Smit et al., 1993), biliary PCs will mix with these ''shorter chain'' SM on the outer hemileaflet of cLPM, which are then eluted as PC(+SM) canalicular vesicles into bile (Möckel et al., 1995).

In summary, the SMs of cLPM and bile displays a bimodal distribution of saturated fatty acid substituents. Since the 16:0 molecular SM species appears in higher concentrations in bile, it is probably miscible with canalicular membrane PC, therefore the more hydrophobic SM molecular species, i.e., 24:1 and 24:0 on the outer

leaflet of the canalicular membranes should be laterally phase-separated since they don't enter bile to the same extent. We can speculate, on the basis of phase equilibria of model systems, that the laterally phase separated SMs will contain higher concentrations of cholesterol molecules, in contrast to canalicular PC. Employing model systems, we (van Erpecum & Carey, 1997) demonstrated that partitioning of submicellar concentrations of bile salts into SM bilayer vesicles destroys this high affinity for cholesterol and forces SM to deliver cholesterol molecules into biliary type PC, when coexisting as vesicles. We speculate that a similar scenario might occur on the canalicular membrane and within the canalicular lumen in the formation of bile. Contrariwise, we would predict that in situations of upregulated biliary PC secretion, the least hydrophobic and miscible 16:0 SMs, may also be eluted in excess into bile.

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