# The role of different P-glycoproteins in hepatobiliary secretion of fluorescently labeled short-chain phospholipids

**Charles M. G. Frijters, Coosje J. Tuijn, Roelof Ottenhoff, Bart N. Zegers, Albert K. Groen, and Ronald P. J. Oude Elferink[1]**

Departments of Gastrointestinal and Liver Diseases and Clinical Chemistry, Academic Medical Center, FO-221, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

**Abstract** **Class III P-glycoproteins (Pgps) mediate biliary phosphatidylcholine (PC) secretion. Recent findings that class I P-glycoproteins are able to transport several short-chain phospholipid analogues raises questions about the role of these Pgps in physiological lipid transport. We investigated the biliary secretion of C6-7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-labeled ceramide and its metabolites in *Mdr1*a/b and *Mdr2* knockout mice compared to control mice. Biliary secretion of these NBD-lipids was unaffected in *Mdr1*a/b −/− mice. Thus neither Mdr1a nor Mdr1b Pgp mediates biliary secretion of these lipids. In contrast, secretion of all three NBD-labeled short-chain phospholipids was significantly reduced in *Mdr2* −/− mice. As in vitro studies revealed that Mdr2 Pgp is not able to translocate these lipid analogues, we hypothesized that *Mdr2* −/− mice had a reduced PC content of the exoplasmic canalicular membrane leaflet so that extraction of the short-chain lipid probes from this membrane by canalicular bile salts was impaired. To investigate this possibility we studied the bile salt-mediated extraction of natural sphingomyelin (SM) and NBD-labeled short-chain SM from small unilamellar vesicles of different lipid composition. Natural SM could be extracted by the bile salt tauroursodeoxycholate from vesicles containing PC, cholesterol (CHOL), and SM (1:2:2) but not from vesicles containing only SM and CHOL (3:2). NBD-labeled short-chain SM could be extracted from vesicles containing PC while its extraction from pure SM:CHOL vesicles was reduced by 65%. These data confirm that the efficiency of NBD-SM extraction depends on the lipid composition and suggest that the canalicular membrane outer leaflet of *Mdr2* −/− mice has a reduced PC content.**—Frijters, C. M. G., C. J. Tuijn, R. Ottenhoff, B. N. Zegers, A. K. Groen, and R. P. J. Oude Elferink. **Role of different P-glycoproteins in hepatobiliary secretion of fluorescently labeled short-chain phospholipids.** ***J. Lipid Res.*** **1999.** 40: **1950–1957.**

**Supplementary key words** NBD-labeled lipids • canalicular membrane • bile formation • hepatobiliary lipid transport • ceramide • sphingomyelin • glucosylceramide • knockout mice • Mdr1a • Mdr1b • Mdr2

In mice, biliary phospholipid secretion is controlled by the canalicular secretion of bile salts and by the activity of Mdr2 P-glycoprotein (Pgp). This ABC-transporter translocates phosphatidylcholine (PC) from the inner leaflet to the outer leaflet of the hepatocyte canalicular membrane, thereby supplying PC molecules for extraction by bile salts in the canalicular space (1–5). The gene products of murine *Mdr1*a and *Mdr1*b and human MDR1, have been identified as amphipathic drug export pumps. Disruption of the *Mdr1*a and *Mdr1*b genes in mice had no effect on the phospholipid composition of bile (6, 7), suggesting that these Pgps are not important for biliary phospholipid secretion. Instead, these Pgps are known to be involved in the biliary secretion of amphipathic drugs (6, 8).

In several studies Pgp-mediated translocation of fluorescent phospholipids across membrane bilayers was studied in vitro. The fatty acid on the *sn*-2 position of these lipids is replaced by a short acyl-chain (6 C-atoms) with 7-nitro-2,1,3-benzoxadiazol-4-yl group (NBD) as a fluorophore. The important advantage of these lipid analogues is that they can be readily inserted into biological membranes by spontaneous lipid transfer from exogenous carriers like bovine serum albumin (BSA) or liposomes. Ruetz and Gros (5) showed an ATP-dependent translocation of NBD-PC in the *Mdr2* transfected yeast but not in *Mdr1*a transfected yeast. Ruetz and Gros (9) and Nies, Gatmaitan, and Arias (10) further demonstrated that this transport can be stimulated by bile salts. Smit et al. (1) provided evidence for translocation of natural PC in intact fibroblasts that express a MDR3 transgene. These results confirmed the proposed PC translocation function of the orthologues Mdr2 Pgp (mouse), Pgp3 (rat), and MDR3 Pgp (human) and they explain the absence of biliary PC in *Mdr2* knockout

Abbreviations: BSA, bovine serum albumin; CHOL, cholesterol; GlucCer, glucosylceramide; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; MRP, multidrug resistance protein; NBD-group, 7-nitro-2,1,3-benzoxadiazol-4-yl group; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pgp, P-glycoprotein; PS phosphatidylserine; SM, sphingomyelin; TUDC, tauroursodeoxycholic acid.

[1] To whom correspondence should be addressed.

mice (1). These mice have no Mdr2 Pgp but do express Mdr1a and Mdr1b Pgp in their canalicular membrane. Van Helvoort et al. (11) loaded MDR1 and MDR3 transfected cells with fluorescent phospholipid precursors like C6-NBD-diacylglycerol or C6-NBD-ceramide and studied the translocation of their metabolites, C6-NBD-(phosphatidylcholine (PC) and phosphatidylethanolamine (PE)) or C6-NBD-(sphingomyelin (SM) and glucosylceramide (GlucCer)) respectively. In their method fluorescent lipids, translocated to the outer membrane leaflet, were extracted using BSA as acceptor in the medium. The outcome of their studies was that human MDR1 and mouse Mdr1a were able to translocate NBD-PC, NBD-SM, and NBD-GlucCer, while the human MDR3 Pgp-mediated translocation was restricted to NBD-PC.

Crawford, Vinter, and Gollan (12) reported on the biliary secretion of NBD-ceramide and its metabolites NBD-GlucCer and NBD-SM in a perfused rat liver system. Similar to endogenous phospholipid secretion, the output of these short-chain phospholipid analogues was bile salt-dependent. From these observation the question rises whether the translocation of labeled short-chain phospholipid analogues by various Pgps is representative for hepatobiliary lipid secretion of natural lipids. Because mouse Mdr1a Pgp appears to be a translocator of NBD-SM, we tested whether Mdr1a or 1b P-glycoproteins control the hepatobiliary secretion of NBD-SM. We studied the secretion of NBD-ceramide and its metabolites in an in situ mouse liver perfusion system using mice with different P-glycoprotein gene disruptions. Because Mdr1a and 1b have overlapping substrate specificity and are both expressed in the canalicular membrane, we used mice in which both genes are disrupted (*Mdr1*a/b −/−) (7). We also used *Mdr2* knockout mice (*Mdr2* −/−) (1), with no hepatobiliary PC secretion and control mice with the same genetic background (FVB) as the knockouts. We studied the bile salt-mediated extraction of SM and its NBD-labeled analogue from membranes of different composition, which may resemble the canalicular membrane surface.

## MATERIALS AND METHODS

### Chemicals

Egg-yolk phosphatidylcholine, cholesterol, N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES), and bovine serum albumin (BSA) (essentially fatty acid-free) were obtained from Sigma (St. Louis, MO). Buttermilk sphingomyelin was obtained from Matreya (Pleasant Gap, PA). Krebs-bicarbonate buffer contained 120 mm NaCl, 24 mm $NaHCO_3$, 1.2 mm $KH_2PO_4$, 4.8 mm KCl, 1.2 mm $MgSO_4$, and 1.3 mm $CaCl_2$; pH 7.4. Tauroursodeoxycholic acid (TUDC, 99%) was obtained from Calbiochem-Novabiochem (La Jolla, CA). Methanol, HPLC-grade, was obtained from J. T. Baker (Deventer, The Netherlands). Triethylamine, 99%, and O-phosphoric acid, 85%, were obtained from Merck (Darmstadt, Germany). C6-NBD-ceramide (6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-sphingosine) and C6-NBD-sphingomyelin (6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-hexanoyl]sphingosylphosphocholine) and C6-NBD-C16-PC (2-(6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine) were obtained from Molecular Probes (Eugene, OR). C6-NBD-glucosyl-ceramide was a kind gift from Dr. G. van Meer, Dept. of Cell Biology and Histology, Academic Medical Center, Amsterdam.

### Preparation of fluorescent lipid probe

C6-NBD-ceramide was bound to bovine serum albumin, adapting the method previously described by Crawford et al. (12). C6-NBD-ceramide (109 nmol dissolved in 25 μl ethanol) was added to 1.25 ml Krebs-bicarbonate, pH 7.45, containing 10 mm HEPES and 30 mg/ml bovine serum albumin (essentially fatty acid-free). After dialyzing the solution 4 times against this medium at 4°C, aliquots were prepared finally containing 6.6 nmol C6-NBD-ceramide bound to bovine serum albumin in a 1:5 molar ratio.

### In situ mouse liver perfusion system

Mouse surgery and perfusion technique and solution preparation were performed as described previously (13, 14). Briefly, under anesthesia (hypnorm (fentanyl/fluanisone), 1 ml/kg, Janssen Pharmaceuticals, Beerse, Belgium and diazepam 10 mg/kg, Roche, Mijdrecht, The Netherlands) the vena cava superior, the gallbladder, and portal vein were cannulated. Perfusion was performed in orthograde direction with Krebs-bicarbonate buffer in a perfusion cabinet thermostatted at 37°C. The perfusion medium was gassed with carbogen (5% $CO_2$, 95% $O_2$, Hoek Loos, Schiedam, the Netherlands) in an oxygenator. Directly after start of the perfusion with Krebs-bicarbonate buffer, tauroursodeoxycholic acid (TUDC) was infused through a three-way connector attached to the portal vein cannula, at a steady rate of 500 nmol × $min^{-1}$ × 100 g body $weight^{-1}$ during the whole experimental period to maintain constant biliary excretion. After a stabilization period of 20 min, a bolus of C6-NBD-ceramide (6.6 nmol) was introduced into the perfusion medium by infusion through the portal vein cannula.

Bile samples were collected at 5-min intervals, diluted ten times with distilled water and immediately frozen at −20°C. C6-NBD-C16-PC was dissolved in eluent (85% 0.45 mm triethylammoniumphosphate in methanol and 15% 0.45 mm triethylammoniumphosphate at pH 3.0) and was used as internal standard. Before injection the samples were diluted four times with internal standard solution that resulted in a final amount of 1.2 pmol per injection.

### Bile and perfusate analysis

Fluorescent lipids were detected using a recently developed reversed-phase HPLC method. The HPLC system used consisted of a Gynkotek 480 HPLC gradient system (Germering, Germany) connected to a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA) with a 20 μl injection loop and a Jasco FP920 Fluorimeter (Tokyo, Japan); excitation wavelength set to 470 nm and emission wavelength on 530 nm. The LC columns (Inertsil ODS-2, 5 μm, 100 mm × 3 mm ID and Spherisorb CN, 3 μm, 100 mm × 4.6 mm ID) used were obtained from Chrompack (Bergen op Zoom, The Netherlands). Data acquisition was performed on a computer with Gynkosoft integration software. The eluent flow-rate was 0.6 ml/min. Gradient runs were performed using (A) 0.45 mm triethylammoniumphosphate at pH 3.0 and (B) 0.45 mm triethylammoniumphosphate in methanol. The initial eluent composition was 15% A and 85% B; after 0.5 min this was linearly changed to 100% B in 9 min and kept for 6 min at 100%. Finally the system was reset to its initial composition in 5.5 min and stabilized for 8 min before the next analysis was started.

### Small unilamellar vesicles

In order to study the behavior of C6-NBD-SM and buttermilk SM in small unilamellar vesicles (SUV) exposed to bile salts, three batches of liposomes with different compositions were prepared. The first batch consisted of PC and CHOL (molar ratio

3:2); the second batch contained PC, CHOL, and SM (molar ratio 1:2:2) and the third batch was prepared by mixing SM and CHOL (3:2). The liposomes were prepared by mixing stock solutions of lipids in chloroform–methanol 4:1 in the indicated ratios and the total amount of lipid in each preparation was 81.25 μmol. All three batches were spiked with NBD-SM (135 pmol). After evaporation of the solvent under a stream of nitrogen, the lipid film was hydrated with 5 ml buffer (10 mm HEPES/135 mm NaCl/0.05% $NaN_3$ (pH 7.4)), and sonicated for 30 min in a Branson B12 probe-type sonicator (80 watts) on ice under a stream of nitrogen. The suspensions were filtered through a sterile 0.45 μm and a 0.22 μm Millex-HA or Millex-GP filter (Millipore, Molsheime, France) subsequently, aliquoted and stored under argon at 4°C for not longer than 3 days.

### Bile salt-mediated lipid extraction assay

Total phospholipid (PC and SM) and cholesterol concentration was determined in the SUV aliquots using the enzymatic methods described in the bile and perfusate analysis section. Just before use, SUV suspensions were diluted to a total lipid concentration of 8.4 mm in 10 mm HEPES/135 mm NaCl/0.05% $NaN_3$ (pH 7.4) containing 10 mm tauroursodeoxycholic acid (TUDC) and incubated for 10 min at 37°C. The vesicles (0.5 ml) were then chromatographed over a Sephacryl S-300 gel permeation column (elution buffer 10 mm HEPES/135 mm NaCl/0.05% $NaN_3$ (pH 7.4) containing 10 mm tauroursodeoxycholic acid) using a flow rate of 0.7 ml/min. Fractions of 2 min were collected for 60 min in which total phospholipid and cholesterol was determined to identify the vesicular fraction and the micellar fraction. Online detection of the fluorescence signal (Jasco FP920 Fluorimeter, Tokyo, Japan) allowed determination of the percentage of NBD-SM fluorescence associated with the vesicular or micellar fraction. The amount of unlabeled SM and PC in the peak fractions comprising the micellar and vesicle fractions (two fractions of 2 min each) was determined by straight-phase HPLC combined with evaporative light scattering detection. Prior to injection on the HPLC column the lipids in the gel permeation column fractions were extracted according to the method described by Bligh and Dyer (15). After evaporation of the organic solvents, the lipid film was dissolved in isopropanol–hexane–water 58:40:2. Essentially the same HPLC set-up was used as described in the bile and perfusate analysis section. Detection was performed using a PL-EMD 960 Evaporative Light Scattering Detector (Polymer Laboratories, Heerlen, The Netherlands). The lipids from the gel permeation fractions were separated using a Chromsphere 3 Si column (150 mm × 3.0 mm ID) obtained from Chrompack (Bergen op Zoom, The Netherlands). The eluent flow-rate was 0.4 ml/min. Gradient runs were performed using (A) isopropanol (B) hexane, and (C) water. The initial eluent composition was 58% A, 40% B, and 2% C; after 0.5 min this was linearly changed to 52% A, 40% B, and 8% C in 7 min and kept for 8 min in this composition. Finally, the system was reset to its initial composition in 5.5 min and stabilized for 8 min before the next analysis was started.

### Statistics

Results are reported as mean ± SD from at least four animals in each experimental group (controls (n = 7), *Mdr2* −/− (n = 4), *Mdr1 a/b* −/− (n=4)). Statistical significance was determined where appropriate by Student's *t*-test for unpaired data.

## RESULTS

### Uptake and metabolism of albumin-bound NBD-labeled ceramide by the liver

The uptake of C6-NBD-ceramide bound to albumin was determined by measuring disappearance of the fluorescence in the perfusate after passage through the liver. Five fractions of 1 min each were collected directly after administration of the single dose of C6-NBD-ceramide followed by 10-min samples. Within the 5-min period directly after administration of C6-NBD-ceramide, a peak in fluorescence was detected; in the later fractions fluorescence was only slightly above background. The rapid peak fraction probably represented the labeled lipid that was not taken up by the liver. HPLC analysis confirmed that in this peak the fluorescent signal was from the C6-NBD-ceramide (data not shown).

The low signal in the later fractions was from NBD-Cer with a small amount of NBD-SM. No NBD-GlucCer was detected in the perfusate during the entire experiment. The uptake of C6-NBD-ceramide by the liver turned out to be very efficient. About 10% of the injected dose of 6.6 nmol C6-NBD-ceramide could be recovered in the perfusion medium after passage through the liver. The uptake values did not differ among the different genotypes. The fractional uptake values for controls, *Mdr1* a/b (−/−) and *Mdr2* (−/−) were 89.7 ± 2.2% (n = 7), 91.9 ± 1.7% (n = 4), and 93.4 ± 1.0% (n = 4) of total administered dose.

### Biliary secretion of NBD-labeled short-chain lipid analogues

The first fluorescent lipid excreted into bile after injection was NBD-C6-ceramide. At 10–15 min after administration a sharp increase in biliary concentration was observed which gradually decreased to low steady state levels (**Fig. 1A**). The bulk of ceramide was excreted within a 50-min period after the administration. In contrast, the metabolites of NBD-C6-ceramide, NBD-C6-glucosylceramide, and NBD-C6-sphingomyelin showed a delayed biliary excretion with kinetics similar to the ceramide (Fig. 1B). The total amount of NBD-labeled lipids secreted during a period of 100 min after administration of the NBD-ceramide was highest in the *Mdr1a/b* (−/−) mice (5.0 ± 1.7% of the administered dose; n = 4). This was, however, not significantly different from the total biliary secretion in control mice (3.7 ± 1.9%; n = 7). Significantly less total NBD-lipid was secreted in *Mdr2* (−/−) mice bile during this period (0.75 ± 0.1%; n = 4). Analysis of bile for the individual fluorescent lipid species revealed that there was no significant difference in biliary secretion between controls and *Mdr1*a/b (−/−) (**Fig. 2**). The 100-min cumulative secretion in controls was: 521.7 ± 313.1 (NBD-ceramide); 123 ± 60 (NBD-glucosylceramide), and 42 ± 21 pmol/100 g body weight (NBD-sphingomyelin). In *Mdr1* a/b (−/−) mice, the secretion values of these lipid species were 748 ± 314, 176 ± 82, and 41 ± 16 pmol/100 g body weight, respectively. However, the fluorescent lipid secretion values in *Mdr2* (−/−) were decreased significantly for all three lipid species. NBD-ceramide values only reached 92 ± 16 pmol/100 g body weight, NBD-glucosylceramide reached 34.0 ± 10 and NBD-sphingomyelin reached 17 ± 3 pmol/100 g body weight.

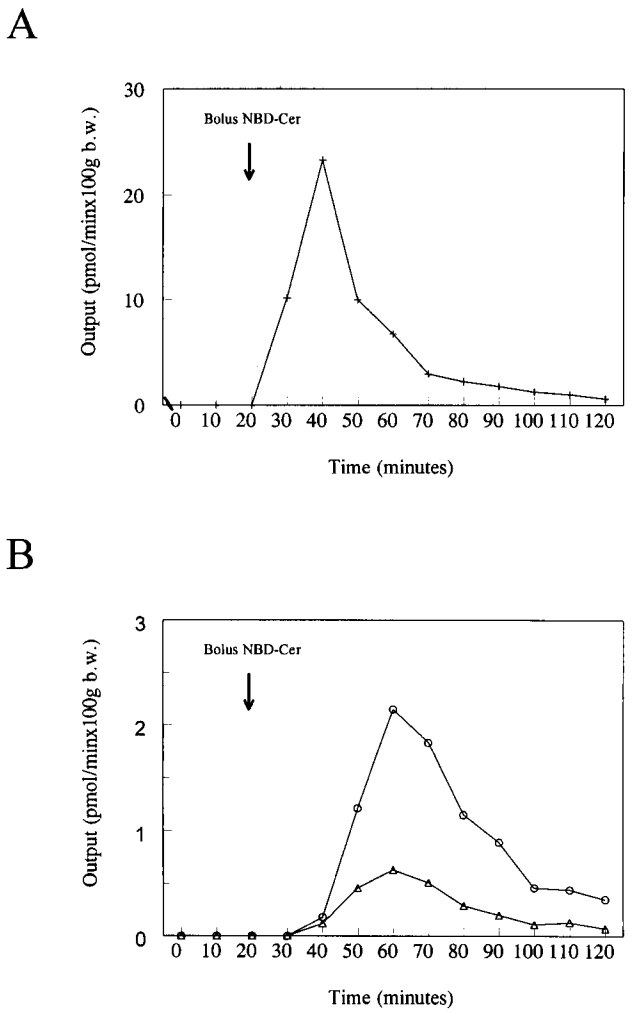


**Fig. 1.** Biliary secretion of NBD-labeled ceramide (panel A) and its metabolites, NBD-sphingomyelin (triangles) and NBD-glucosylceramide (circles) (panel B) in a perfused control mouse liver. After a 20-min steady-state perfusion period, NBD-ceramide was administered in a single dose to the perfusion medium. The mouse liver was perfused for a total of 120 min. Bile fractions were collected and analyzed by HPLC as described in Materials and Methods. Output levels are expressed in pmol/min and normalized to 100 g body weight.

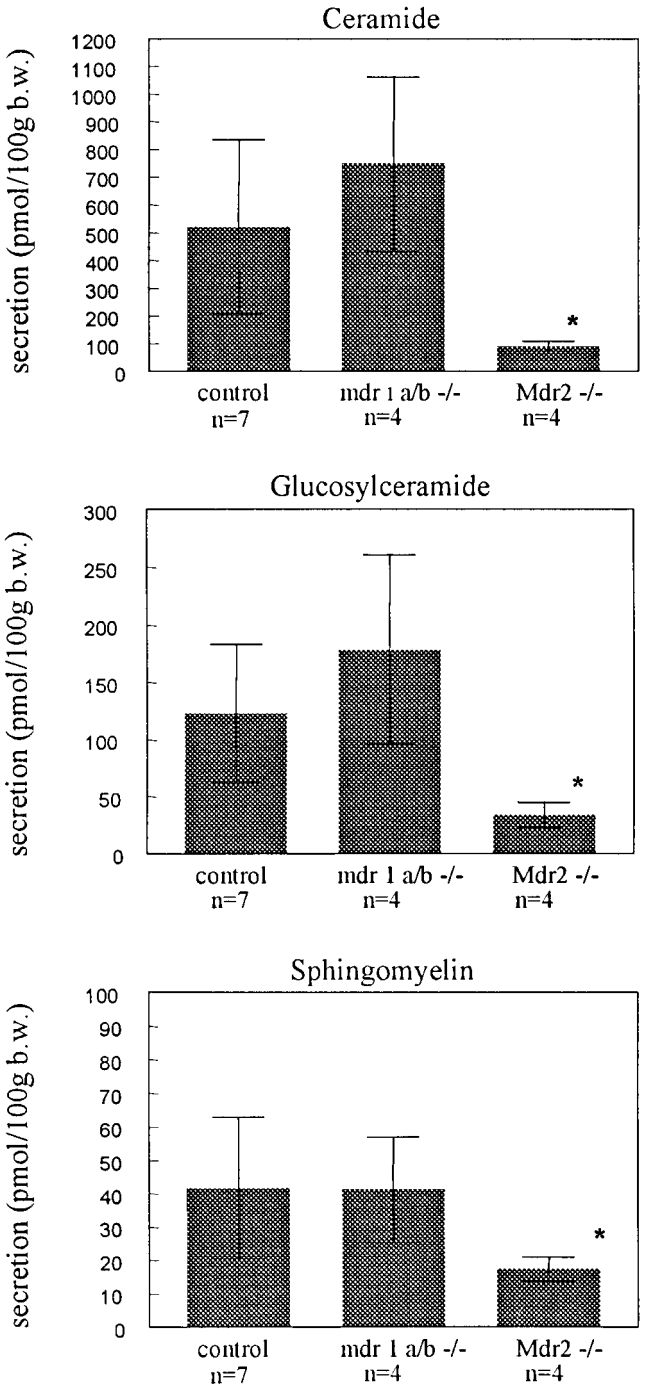


**Fig. 2.** Biliary secretion of NBD-labeled lipids in mice with different P-glycoprotein genotypes. The values represent the cumulative fluorescence in bile during the 100-min post-administration of NBD-ceramide (error bars indicate standard deviation). Values from Mdr2 (−/−) and Mdr1 a/b (−/−) mice were tested for statistically significant differences with data from control mice by Student's *t*-test; * $P < 0.05$.

### Extraction of natural and short-chain NBD-labeled SM from lipid vesicles by tauroursodeoxycholate

The reduced NBD-lipid secretion observed in *Mdr2* (−/−) mice was somewhat unexpected because these lipids are not substrates for Mdr2 Pgp and this reduction could be a secondary effect of the absence of Mdr2 Pgp. The outer membrane leaflet of canalicular membranes probably contains phosphatidylcholine, sphingomyelin, and cholesterol. It is expected that, due to the absence of supply by Mdr2 Pgp, the PC content of this membrane leaflet in *Mdr2* (−/−) mice is drastically reduced. We hypothesized that such alteration of the lipid composition of the canalicular outer membrane leaflet in *Mdr2* −/− mice causes reduced bile salt-mediated NBD-lipid extraction. To test this hypothesis, we compared the behavior of buttermilk SM and NBD-labeled short-chain SM in membranes with different lipid composition exposed to bile salts, in an in vitro extraction assay. Briefly, lipid vesicles were prepared containing PC-CHOL (3:2 molar ratio), PC-SM-CHOL (1:2:2), or SM-CHOL (3:2) and trace amounts of NBD-SM were added to all. The vesicles were preincubated for 10 min at 37°C with tauroursodeoxycholate (10

mm) and then applied to a gel permeation column, which was eluted with a buffer containing 10 mm TUDC. Analysis of the eluted fractions for both NBD-labeled and non-labeled lipid species revealed the fractional distribution of lipid over the micellar and vesicular fraction.

**Figure 3** shows the online detection of fluorescent signal and the amount of cholesterol and phospholipid in fractions collected from the gel permeation column after application of the SUVs of different composition in TUDC. The lipids eluted in two peak fractions. The first peak contains the large vesicular structures (vesicular peak) and the second peak contains the smaller micelles (micellar peak).

As expected, the amount of lipids ending up in the mi-

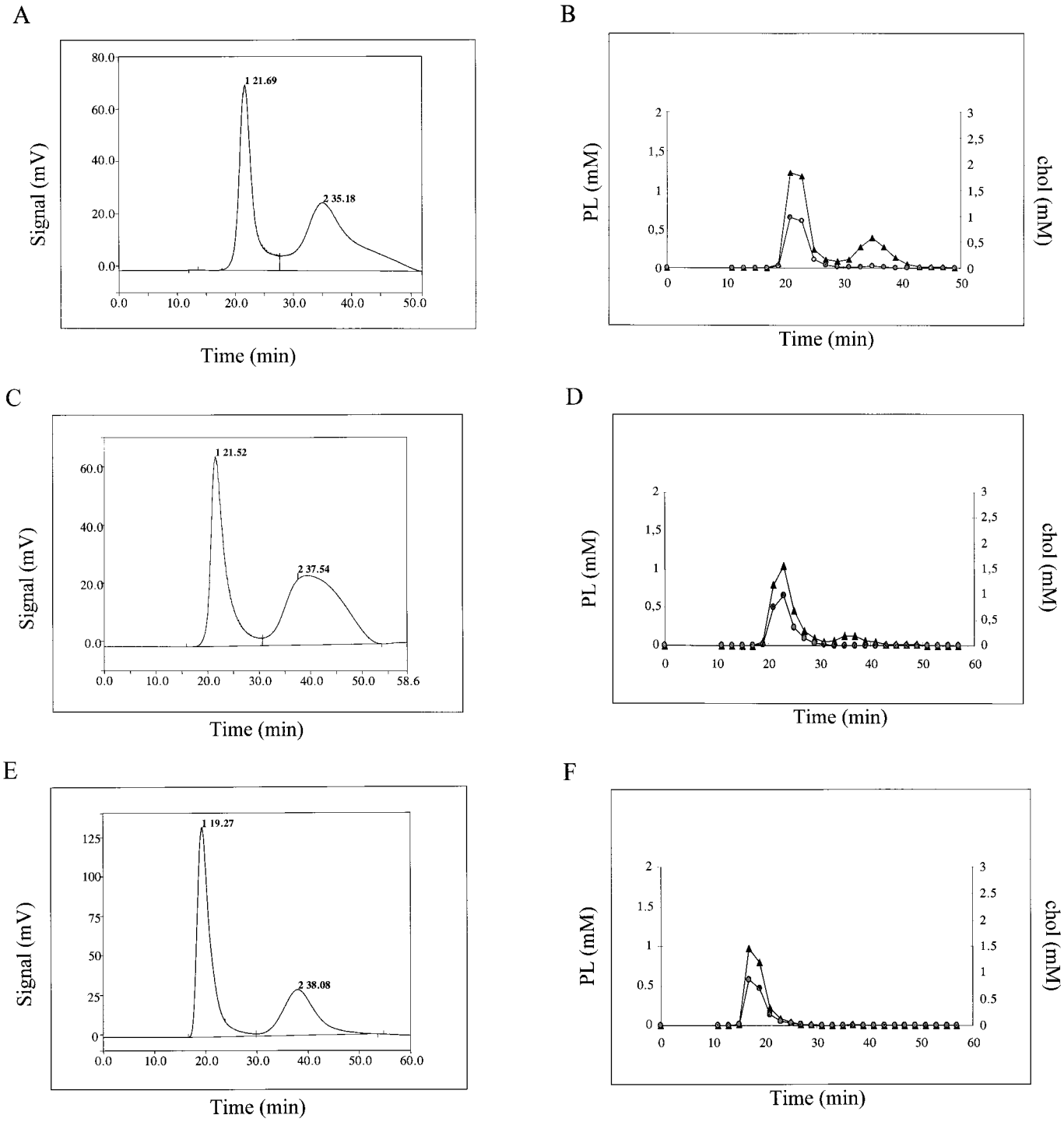


**Fig. 3.** Separation of lipid vesicles and mixed micelles by gel permeation after incubation of small unilamellar vesicles (SUVs) with 10 mm TUDC. Panels B, D, and F reflect the cholesterol (circles) and total phospholipid (PC + SM) (triangles) concentration in the collected fractions. Panels A, C, and E reflect the online registration of fluorescence of NBD-SM that was added in trace amounts to all vesicles. SUVs of different composition were tested: PC-CHOL (panels A and B), PC-CHOL-SM (panels C and D), and CHOL-SM (panels E and F), respectively.

cellar peak varied with the composition of the vesicles. PL and cholesterol were best extracted from SUVs composed of PC-CHOL; about 15–20% of total amount of lipid that was loaded on the column was recovered in the micellar fraction (Fig. 3B). When SM was also present in the SUVs (PC:SM:CHOL (1:2:2)) the amount of lipids retrieved in the micellar peak was only 4–5% of the total amount loaded on the column (Fig. 3D). In SUV containing exclusively SM and CHOL, no phospholipid (i.e., SM) and cholesterol could be detected in the micellar peak (Fig. 3E). Because the enzymatic assay that was used could not distinguish between PC and SM, we analyzed the main peak fractions with HPLC and evaporative light scattering detection. These data showed that in case of PC-SM-CHOL vesicles, the micellar fraction predominantly contained PC. The ratio PC:SM in the vesicular peak was 3.5 ± 0.6 times lower than in the micellar peak. The data also confirmed the absence of SM in the micellar fraction when the SM-CHOL vesicles were used. The online detection of NBD-SM extracted by bile salt from the different SUVs is depicted in Fig. 3A, C, and E, respectively, and showed a behavior that was markedly different from the natural long-chain buttermilk SM. In contrast to natural SM, the extraction of NBD-SM was equally high in PC-CHOL- and PC-SM-CHOL-containing vesicles. The most striking difference was the identification of NBD-SM-associated fluorescence in the micellar fraction in CHOL-SM vesicles. Although extraction from these vesicles was significantly reduced compared to the SUVs containing PC, still 35% of the NBD-SM could be retrieved in the micelles (**Fig. 4**).

## DISCUSSION

Although the mechanism of biliary phospholipid secretion is not fully understood, two major factors driving this process can be distinguished. First, activity of Mdr2 P-glycoprotein in the canalicular membrane is an absolute requirement for phospholipid secretion. Second, bile salt secretion into the canalicular lumen is necessary to evoke lipid secretion and the efficiency of this extraction increases with hydrophobicity of the bile salt species. These observations have been combined in a hypothetical model describing the mechanism of lipid secretion (reviewed in refs. 3, 4, 16). In short, PC molecules are delivered to the canalicular membrane leaflet facing the cytoplasm. Mdr2 Pgp (MDR3 in humans) translocates these molecules in an ATP-driven process to the leaflet facing the canalicular lumen where they vesiculate in biliary vesicles. The latter process requires bile salts to be present in the canalicular lumen.

Murine Mdr1a and human MDR1 Pgp are capable of translocating short-chain phospholipids but not natural long-chain PC (11). In their study, van Helvoort et al. (11) found that these class I Pgps translocate NBD-SM, NBD-GlucCer, NBD-Cer as well as NBD-PC while MDR3 Pgp (the human orthologue of murine Mdr2) only translocated NBD-PC. In addition, Crawford et al. (12) reported that NBD-SM and NBD-GlucCer are secreted into bile of the isolated perfused rat liver in a bile salt-dependent fashion. From these observations the question rose as to which role class I Pgps play in the biliary secretion of phospholipids other than PC. It is unlikely that class I Pgps play any quantitative role in the biliary secretion of natural PC, because the secretion of this phospholipid is virtually abrogated in *Mdr2* knockout mice, while these animals do express Mdr1a and 1b. These animals actually have a strongly induced expression of these P-glycoproteins compared to wild-type animals (1). The reason for this is unknown but might be related to the liver pathology.

To investigate the role of class I Pgps in the biliary secretion of other phospholipids, we studied the biliary secretion of NBD-ceramide, NBD-SM, and NBD-GlucCer in perfused livers from *Mdr1*a/b double knockout mice. Because both Pgps are very similar in drug transport activity and both are expressed in the canalicular membrane, it was essential to use this double knockout. The liver perfusion technique with C6-NBD-ceramide as described by Crawford (12) was successfully adapted for use in mice. In contrast to Crawford et al., who administered NBD-ceramide incorporated in liposomes, we added the lipid to the perfusate in an albumin-bound form. It is quite unlikely that this leads to a significantly different outcome of the experiments; in both methods the applied NBD-lipid is taken up very efficiently.

We used a recently developed HPLC method for the

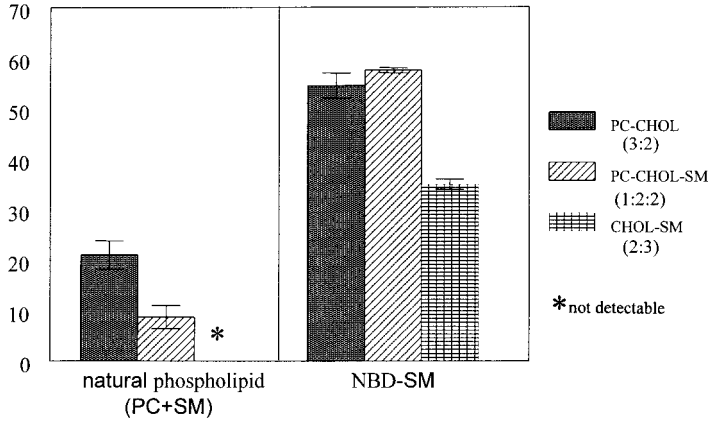


**Fig. 4.** Extraction of phospholipid by TUDC from vesicles of different composition. The data are the means and standard deviations (indicated by error bars) of three separate experiments with SUV of different lipid composition: PC-CHOL (3:2) (left bar); PC-SM-CHOL (1:2:2) (middle bar) and SM-CHOL (3:2) (right bar). Left panel shows the amount of phospholipid (PC and SM; enzymatically detected) that is retrieved in the micellar fraction as percentage of total phospholipid loaded onto the column. Right panel displays the fraction of NBD-labeled SM that associates with the micellar fraction as percentage of total NBD-SM loaded onto the gel-permeation column.

identification and quantification of NBD-lipids in bile and perfusate samples (14). About 90% of the administered dose of NBD-ceramide was taken up by the liver with no difference between the mouse genotypes that were used. After first passage of the injected material, only tiny amounts of NBD-lipid are secreted into the perfusate during the remainder of the experiment. Further analysis showed that this was mainly NBD-Cer and some NBD-SM. The hydrophilic nature of NBD-Cer allows this lipid to migrate easily through the cytosol and undergoes rapid equilibration between all cellular membranes. Therefore it is not unexpected that NBD-Cer is released at low concentrations from the basolateral membrane into the perfusion medium. The appearance of small amounts of the NBD-Cer metabolite NBD-SM but not NBD-GlucCer in the perfusion medium is in agreement with the cellular compartmentalization of the enzymes involved. The enzyme responsible for biosynthesis of sphingomyelin from the precursor ceramide, sphingomyelin synthase, is predominantly localized in the Golgi and to a lesser extent at the exoplasmic leaflet of the plasma membrane (17, 18). Exogenous addition of NBD-Cer in the liver perfusion most likely provides enough substrate for the SM-synthase at the exoplasmic membrane leaflet to produce NBD-SM that is partly recovered in the perfusion medium. On the other hand, glucosyltransferase, involved in the synthesis of glucosylceramide, is located at the cytosolic site of the *cis*-Golgi and some other Golgi-related compartments (19, 20). The NBD-GlucCer produced will diffuse through the cytoplasm to the plasma membrane but there it can only enter the cytoplasmic oriented membrane leaflet. Spontaneous flipping from this leaflet to the exoplasmic membrane leaflet is slow and therefore no or very low concentrations of NBD-GlucCer can be found in the perfusion medium.

The above-mentioned rapid equilibration of NBD-Cer with all cellular membranes probably contributes to the more rapid clearance kinetics of this lipid as opposed to the metabolites. Transport of exogenous NBD-Cer to the site of biosynthesis and subsequent transfer of the metabolites to the canalicular membrane also play a part in the delayed appearance in bile. The biliary secretion kinetics of NBD-C6-ceramide and its metabolites in mice in this study is very similar to that described by Crawford et al. for rat liver (12). From our study it is clear that the hepatobiliary secretion of short-chain NBD-labeled ceramide and its metabolites C6-NBD-SM and C6-NBD-GlucCer is not significantly influenced by the absence of Mdr1a and Mdr1b P-glycoproteins. This shows that class I Pgps do not play a role in the biliary secretion of these lipids. It is not clear whether these lipids require a translocator for secretion into bile, but if they do, it remains to be determined which translocator this is. Recent publications using fluorescently labeled analogues of phosphatidylserine (PS) and PC suggest that the outward directed transport of these lipids in human and murine red blood cells is mediated by Multidrug Resistance Protein 1 (MRP1) (21, 22). Analysis of NBD-lipid flipping in red blood cells from *Mdr1*a −/−, *Mdr2* −/−, and *Mrp1* −/− mice revealed that in these cells Mrp1 is responsible for the observed lipid translocation. Furthermore, the inhibitory effect of several drugs on this translocating activity correlated closely with the characteristics observed for MRP-like transporters and correlated poorly with the inhibiting effect on active transport mediated by MDR1 P-glycoprotein (22). These data are in full agreement with our observation that biliary secretion of NBD-labeled lipids is not affected in Mdr1 a/b −/− and supports our hypothesis that the reduced biliary secretion in *Mdr2* −/− mice is secondary to the absence of Mdr2 Pgp. We cannot rule out that members of the MRP-subfamily (for instance MRP-2) are responsible for the biliary lipid secretion in control and *Mdr1 a/b* (−/−) mice.

An unexpected finding was the reduced secretion of NBD-Cer, NBD-GlucCer, and NBD-SM in *Mdr2* −/− mice, because these lipids are not substrates for Mdr2 Pgp (11). To rule out the possibility that the biosynthesis of NBD-GlucCer and NBD-SM was altered in *Mdr2* −/− mice, we analyzed the NBD-lipids present in perfused livers of both controls and *Mdr2* −/− mice at 40 min after administration of NBD-Cer. No difference in the amount of NBD-lipids could be detected. Therefore the most likely explanation of the reduced NBD-lipid secretion is an effect secondary to the absence of Mdr*2* Pgp. The absence of mdr2 Pgp from the canalicular membrane might lead to a reduction of the PC content and as a consequence to a relative increase in the SM content. It is extremely difficult to analyze lipid asymmetry in canalicular membranes in situ. To circumvent this problem we studied bile salt (TUDC)-mediated extraction of SM and NBD-SM from lipid vesicles with different outer membrane lipid content.

The major bile salt species found in normal mouse bile are muricholic acid (80%) and to a lesser extent taurocholic acid (20%) (23). In our infusion and extraction experiments we used TUDC because muricholic acid is not available in sufficient quantities and TUDC is the only other bile salt suitable to infuse at high fluxes without generation of cholestatic effects. We observed that exchanging PC in the vesicles with SM led to a dramatic reduction of the extraction of phospholipids by TUDC. This effect was reported previously, directly or indirectly, in several experimental set-ups (24–26). In vesicles composed of both PC and SM, much more PC than SM was extracted by the bile salt. The low extraction efficiency of SM is probably caused by the strong tendency of cholesterol to bind SM (27–29), by the fact that (buttermilk) SM contains more saturated fatty acids than egg yolk PC, and by the fact that sphingolipids form hydrogen bonds with each other via the free OH group in the sphingosine base. We used buttermilk SM and egg yolk PC because these resemble SM and PC from the canalicular membrane (30). In vesicles containing only SM and cholesterol, no phospholipid (SM) extraction into micelles was observed at all. In contrast to natural (long-chain) SM, which was not extracted from these vesicles at all, NBD-SM still underwent significant extraction from CHOL:SM vesicles, albeit at a reduced level. While extraction of NBD-SM from PC-CHOL and PC-SM-CHOL vesicles was identical, it was reduced by

65% in vesicles containing only SM and CHOL. These observations demonstrate two important characteristics of NBD-SM. First, it clearly shows the different behavior of NBD-SM from natural (buttermilk) SM. The latter is resistant towards bile salt-mediated extraction, while the former is extracted quite well, even from very resistant vesicles such as those exclusively composed of SM and CHOL. Second, the reduced extraction of NBD-SM from SM-CHOL vesicles as compared to PC-SM-CHOL vesicles may suggest that the canalicular membrane of *Mdr2 −/−* hepatocytes is relatively more rich in SM than that in control hepatocytes due to the absence of Mdr2 Pgp-mediated PC translocation. This, however, remains to be proven.

This work was supported by grant 902-23-097 by the Dutch Organisation of Scientific Research. The authors wish to thank Dr. G. van Meer for donating NBD-glucosylceramide standard and B. Delzenne for his expert help with the HPLC analysis.

*Manuscript received 31 March 1999 and in revised form 8 July 1999.*

## REFERENCES

1. Smit, J. J. M., A. H. Schinkel, R. P. J. Oude Elferink, A. K. Groen, E. Wagenaar, L. van Deemter, C. A. A. M. Mol, R. Ottenhoff, N. M. T. Van der Lugt, M. A. van Roon, M. A. Van der Valk, G. J. A. Offerhaus, A. J. M. Berns, and P. Borst. 1993. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell.* **75:** 451–462.
2. Oude Elferink, R. P., and A. K. Groen. 1995. The role of mdr2 P-glycoprotein in biliary lipid secretion. Cross-talk between cancer research and biliary physiology. *J. Hepatol.* **23:** 617–625.
3. Frijters, C. M. G., A. K. Groen, and R. P. J. Oude Elferink. 1997. Mdr2 p-glycoprotein-mediated lipid secretion and its relevance to biliary drug transport. *Adv. Drug Delivery Rev.* **25:** 201–215.
4. Oude Elferink, R. P. J., R. Ottenhoff, M. J. A. Van Wijland, J. J. M. Smit, A. H. Schinkel, and A. K. Groen. 1995. Regulation of biliary lipid secretion by mdr2-P-glycoprotein in the mouse. *J. Clin. Invest.* **95:** 31–38.
5. Ruetz, S., and P. Gros. 1994. Phosphatidylcholine translocase: A physiological role for the mdr2 gene. *Cell.* **77:** 1071–1081.
6. Schinkel, A. H., J. J. M. Smit, O. Van Tellingen, J. H. Beijnen, E. Wagenaar, L. van Deemter, C. A. A. M. Mol, M. A. Van der Valk, E. C. Robanus-Maandag, H. P. J. Teriele, A. J. M. Berns, and P. Borst. 1994. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell.* **77:** 491–502.
7. Schinkel, A. H., U. Mayer, E. Wagenaar, C. A. Mol, L. van Deemter, J. J. Smit, M. A. Van der Valk, A. C. Voordouw, H. Spits, O. Van Tellingen, J. M. Zijlmans, W. E. Fibbe, and P. Borst. 1997. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) p-glycoproteins. *Proc. Natl. Acad. Sci. USA.* **94:** 4028–4033.
8. Smit, J. W., A. H. Schinkel, M. Muller, B. Weert, and D. K. Meijer. 1998. Contribution of the murine mdr1a p-glycoprotein to hepatobiliary and intestinal elimination of cationic drugs as measured in mice with an mdr1a gene disruption. *Hepatology.* **27:** 1056–1063.
9. Ruetz, S., and P. Gros. 1995. Enhancement of mdr2-mediated phosphatidylcholine translocation by the bile salt taurocholate: implications for hepatic bile formation. *J. Biol. Chem.* **270:** 25388–25395.
10. Nies, A. T., Z. Gatmaitan, and I. M. Arias. 1996. Atp-dependent phosphatidylcholine translocation in rat liver canalicular plasma membrane vesicles. *J. Lipid Res.* **37:** 1125–1136.
11. van Helvoort, A., A. J. Smith, H. Sprong, I. Fritzsche, A. H. Schinkel, P. Borst, and G. Van Meer. 1996. Mdr1 p-glycoprotein is a lipid translocase of broad specificity, while mdr3 p-glycoprotein specifically translocates phosphatidylcholine. *Cell.* **87:** 507–517.
12. Crawford, J. M., D. W. Vinter, and J. L. Gollan. 1991. Taurocholate induces pericanalicular localization of C6-NBD- ceramide in isolated hepatocyte couplets. *Am. J. Physiol.* **260:** G119–G132.
13. Groen, A. K., M. J. Van Wijland, W. M. Frederiks, J. J. Smit, A. H. Schinkel, and R. P. Oude Elferink. 1995. Regulation of protein secretion into bile: studies in mice with a disrupted mdr2 p-glycoprotein gene. *Gastroenterology.* **109:** 1997–2006.
14. Frijters, C. M. G., C. J. Tuijn, F. J. Hoek, A. K. Groen, R. P. J. O. Elferink, and B. N. Zegers. 1998. Reversed-phase liquid chromatographic method for the determination of 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labelled lipid analogues. *J. Chromatogr. B.* **710:** 9–16.
15. Bligh, E. G., and Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
16. Oude Elferink, R. P. J., G. N. J. Tytgat, and A. K. Groen. 1997. The role of mdr2 P-glycoprotein in hepatobiliary lipid transport. *FASEB J.* **11:** 19–28.
17. Jeckel, D., A. Karrenbauer, R. Birk, R. R. Schmidt, and F. Wieland. 1990. Sphingomyelin is synthesized in the *cis* Golgi. *FEBS Lett.* **261:** 155–157.
18. Van Helvoort, A., W. Stoorvogel, G. Van Meer, and N. J. Burger. 1997. Sphingomyelin synthase is absent from endosomes. *J. Cell Sci.* **110:** 781–788.
19. Futerman, A. H., and R. E. Pagano. 1991. Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver. *Biochem. J.* **280:** 295–302.
20. Jeckel, D., A. Karrenbauer, K. N. Burger, G. Van Meer, and F. Wieland. 1992. Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. *J. Cell Biol.* **117:** 259–267.
21. Dekkers, D. W., P. Comfurius, A. J. Schroit, E. M. Bevers, and R. F. Zwaal. 1998. Transbilayer movement of NBD-labeled phospholipids in red blood cell membranes: outward-directed transport by the multidrug resistance protein 1 (MRP1). *Biochemistry.* **37:** 14833–14837.
22. Kamp, D., and C. W. Haest. 1998. Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane. *Biochim. Biophys. Acta.* **1372:** 91–101.
23. Frijters, C. M., R. Ottenhoff, M. J. Van Wijland, C. M. Van Nieuwkerk, A. K. Groen, and R. P. Oude Elferink. 1997. Regulation of mdr2 p-glycoprotein expression by bile salts. *Biochem. J.* **321:** 389–395.
24. Caderni, G., E. Stuart, A. M. Anderson, and W. R. Bruce. 1987. A cho cell line resistant to deoxycholic acid. *Cancer Lett.* **38:** 119–127.
25. Coleman, R., P. J. Lowe, and D. Billington. 1980. Membrane lipid composition and susceptibility to bile salt damage. *Biochim. Biophys. Acta.* **599:** 294–300.
26. Van Erpecum, K. J., and M. C. Carey. 1997. Influence of bile salts on molecular interactions between sphingomyelin and cholesterol: relevance to bile formation and stability. *Biochim. Biophys. Acta.* **1345:** 269–282.
27. Porn, M. I., M. P. Ares, and J. P. Slotte. 1993. Degradation of plasma membrane phosphatidylcholine appears not to affect the cellular cholesterol distribution. *J. Lipid Res.* **34:** 1385–1392.
28. Ohvo, H., C. Olsio, and J. P. Slotte. 1997. Effects of sphingomyelin and phosphatidylcholine degradation on cyclodextrin-mediated cholesterol efflux in cultured fibroblasts. *Biochim. Biophys. Acta.* **1349:** 131–141.
29. Chatterjee, S. 1993. Neutral sphingomyelinase increases the binding, internalization, and degradation of low density lipoproteins and synthesis of cholesteryl ester in cultured human fibroblasts. *J. Biol. Chem.* **268:** 3401–3406.
30. Nibbering, C. P., and M. C. Carey. 1999. Sphingomyelins of rat liver: Biliary enrichment with molecular species containing 16 : 0 fatty acids as compared to canalicular-enriched plasma membranes. *J. Membr. Biol.* **167:** 165–171.