Biliary lipid secretion: immunolocalization and identification of a protein associated with lamellar cholesterol carriers in supersaturated rat and human bile[1]

Attilio Rigotti,§ Liliana Núñez,* Ludwig Amigo,* Luigi Puglielli, Jorge Garrido,§ Manuel Santos,§ Sergio González,† Geltrude Mingrone,** Aldo Greco,** and Flavio Nervi[2,*]**

Departamentos de Gastroenterología,* Anatomía Patológica,† y Biología Celular y Molecular,§ Pontificia Universidad Católica, Santiago, Chile; and Istituto di Clinica Medica,** Universitá Cattolica del Sacro Cuore, Roma, Italia

Abstract Feeding a 0.5% diosgenin plus 0.02% simvastatin diet to rats increases biliary cholesterol concentration and saturation to levels generally found in human native supersaturated bile. By using preparative ultracentrifugation, gel filtration chromatography, and electron microscopy, we isolated, purified, and identified lamellar structures (unilamellar vesicles and multilamellae) as a major biliary cholesterol transport in supersaturated human and rat bile. It was estimated that more than 60% of biliary cholesterol is transported in these lamellar carriers, which were identified by transmission electron microscopy as unilamellar vesicles and multilamellar bodies within bile canaliculi of rats with cholesterol supersaturated bile. By SDS-PAGE, a characteristic and constant protein profile was found associated to the purified lamellar carriers. One of these proteins, a 130-kDa protein, was isolated from human biliary lamellae and used for preparation of a rabbit polyclonal antibody, which cross-reacted with the homologous rat protein. By Western blotting, it was established that the purified low density fraction of bile-Metrizamide gradients, containing lamellae, was enriched with the 130-kDa protein. The 130-kDa protein was characteristically detected at the canalicular membrane by Western blotting of hepatic subcellular fractions and by immunohistochemistry of rat and human liver biopsies. Amino acid sequencing of the amino terminus of the 130-kDa protein demonstrated a complete identity with aminopeptidase N, a canalicular transmembrane hydrophobic glycoprotein. These studies show that biliary lipids may acquire an ordered multilamellar structure that is present in the canaliculi of rats with supersaturated bile. These biliary lamellae are similar to lamellar bodies and surfactant-like material frequently found in other epithelia, suggesting common biogenetic, structural, and functional properties. The identification of aminopeptidase N associated with biliary lamellae is consistent with the involvement of the canalicular membrane in the secretory mechanism of biliary lipids.—**Rigotti, A., L. Núñez, L. Amigo, L. Puglielli, J. Garrido, M. Santos, S. González, G. Mingrone, A. Greco, and F. Nervi.** Biliary lipid secretion: immunolocalization and identification of a protein associated with lamellar cholesterol carriers in supersaturated rat and human bile. *J. Lipid Res.* 1993. **34:** 1883–1894.

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The cellular and molecular mechanisms of biliary lipid secretion are poorly understood. Originally, it was considered that biliary cholesterol was entirely carried in bile salts and lecithin mixed micelles (1, 2). However, more recent chromatographic, ultracentrifugal, and ultrastructural data have demonstrated that native biliary cholesterol may be solubilized and transported in mixed micelles, or in both unilamellar vesicles and multilamellae (3–10). It has been postulated that biliary cholesterol and phospholipids are primarily secreted in unilamellar lipid complexes from the hepatocyte into the canalicular lumen (11–14). Different theoretical models have been proposed for the transport of biliary lipids through the canalicular membrane, including exocytosis, membrane shedding, and the fusion-budding model of the canalicular membrane (12, 14). However, there is, so far, scarce experimental evidence to support some of these models. In vitro model systems of biliary lipid aggregations have been used to explore the possible early physicochemical events that occur during biliary lipid secretion (15). These observations are limited because they derive from artificial lipid systems, which exclude the potentially important effects of physiological bile salts, lecithins, and non-lipid biliary components, such as proteins.

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[2]To whom correspondence should be addressed at: Departamento de Gastroenterología, Casilla 114-D, Santiago, Chile.

The major aspects related to the nature and biogenesis of the biliary cholesterol non-micellar carriers can only be answered by isolating them in their native state and by recognizing specific marker constituents, such as proteins and lipids. The existence of biliary proteins specifically associated to biliary lipid lamellar carriers has been controversial (16, 17). There has been no clear evidence of some specific role of these proteins in the secretion and/or transport of biliary lipids (16, 17). A major problem is related to the technical difficulties of isolating and purifying these carriers. We have recently isolated native cholesterol-rich biliary vesicles by ultracentrifugation of human hepatic bile and then purified them by sequential gel filtration chromatography (18). These vesicles were mainly composed of cholesterol and phospholipids, but they also contained a unique and constant hydrophobic glycoprotein profile (19). These results allowed us to speculate that some of these vesicular proteins could be co-secreted with biliary lipids and that they might be used as markers to analyze in detail the cellular process related to biliary lipid secretion.

This study was undertaken to disclose some of the cellular mechanisms of biliary lipid secretion, as related to the biogenesis of the non-micellar cholesterol carriers. First, we characterized, by biochemical and ultrastructural techniques, an experimental model of lamellar (unilamellar vesicles and multilamellae) biliary lipid secretion in the rat. This model presented a biliary lipid composition and cholesterol saturation similar to that found in native human bile. The major biliary cholesterol carriers were isolated, purified, and characterized in terms of lipid and protein composition. Second, a polyclonal antibody against the 130-kDa lamellae-associated protein was used to determine its differential association to biliary lipid carriers and its subcellular distribution and localization, both in rat and human liver. Finally, the 130-kDa biliary protein was identified by amino acid sequencing and by Western blotting.

MATERIALS AND METHODS

Materials

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): diosgenin, thymerosal, PMSF, aprotinin, leupeptin, Metrizamide (2-[3-acetamido-5-N-methylacetamino-2,4,6-triodobenzamido]-2-deoxy-D-glucose), pentobarbital, glycine, acrylamide, polyacrylamide, Freund's adjuvant, Tween-20, Protein A conjugated to alkaline phosphatase, Sepharose CL-2B-300, sodium taurocholate, 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, rabbit peroxidase-anti-peroxidase, carrageenan, rabbit anti-human albumin IgG antiserum, goat anti-rabbit IgG antiserum, 3,3' diaminobenzidine, 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS), and synthetic phospholipids. Polyethylene catheters were purchased from Clay Adams Inc. (Parsippany, NJ). One-μm pore-size filters and polyvinylidene difluoride membranes were obtained from Millipore (Bedford, MA). 1,2-[^{3}H]cholesterol was purchased from New England Nuclear (Boston, MA). Ultrogel AcA-34 was obtained from Pharmacia LKB (Piscataway, NJ). Centricon concentrators were purchased from Amicon (Danvers, MA). Nitrocellulose membranes were obtained from Amersham (Arlington Heights, IL). Sucrose, sodium azide, EDTA, Tris-HCl, trichloroacetic acid, silica gel G plates, and all organic solvents were purchased from E. Merck (Darmstadt, Germany). Simvastatin was kindly provided by Merck Sharp & Dohme (Rahway, NJ).

Human studies

The experimental protocols were approved by the Ethical Committee for Research of the Facultad de Medicina of the Pontificia Universidad Católica de Chile. In some experiments, a fraction of the tissue removed in liver biopsies was used after written informed consent from the patients. Only liver tissue with minor histological abnormalities was used.

Animals and diet

Male Wistar rats (100–120 g) were subjected to reversed light cycling for 2–3 days before use. The mid-dark point was set at 10 AM. Biliary cholesterol secretion was stimulated by feeding the animals ground chow that contained (wt/wt) 0.5% diosgenin plus 0.02% simvastatin for 1 week. Diosgenin and simvastatin were dissolved in chloroform, mixed with diet, and the solvent was evaporated over a 24-h period under a hood.

Bile sampling

On the day of the experiments, rats were anesthetized with intraperitoneal pentobarbital (4.5 mg/100 g body wt) at 8–9:30 AM. The common bile duct was cannulated with PE10 polyethylene catheter and bile specimens were obtained during the first 30 min of bile fistula for basal biliary lipid and protein determination. Then bile was collected for 3–4 h in sterile tubes with 0.05% chloramphenicol, 3 mM sodium azide, 0.2 mM thymerosal, 5 mM EDTA, and 1 mM PMSF as preservatives. Bile samples from different animals were pooled and stored at 4°C until processed for separation of biliary lipid carriers on the same day of the experiment.

For comparison with rat bile, human native hepatic bile was collected under the same conditions described above from cholesterol gallstone patients with indwelling T-tubes 5 days after cholecystectomy and choledocotomy. Hepatic bile was immediately transported to the laboratory for processing exactly as rat bile.

Isolation and purification of native non-micellar biliary lipid carriers

Non-micellar carriers of biliary cholesterol were isolated by a rapid ultracentrifugation method (20). To discard particulate materials, bile samples were first centrifuged at 10,000 rpm for 10 min at 10°C in a Sorvall centrifuge (DuPont Instruments, Wilmington, DE) and filtered through 1-μm pore-size filters. Then bile was incubated with [^{3}H]cholesterol (0.25 μCi/ml, sp act 44.3 μCi/mmol) for 1 h at 37°C to radiolabel the biliary lipid carriers for their relative quantitation and to follow them during the purification steps. Then 16% Metrizamide was directly dissolved in bile. The bile–Metrizamide mixture was centrifuged at 50,000 rpm for 135 min at 10°C in a TV-865 Sorvall rotor (DuPont Instruments, Wilmington, DE) for separation of lipid carriers in a continuous density gradient. Gradient fractions were collected from the centrifuge tubes by puncturing and the density of the fractions was measured by the method of Beaufay et al. (21). The relative proportion of the different cholesterol carriers in native bile was determined as previously described (20).

The white opalescent band from the low density fraction of the gradients was further purified by two sequential gel filtration chromatography steps as previously described (18). The elution profiles of the biliary lipid carriers were detected by [^{3}H]cholesterol liquid scintillation counting. First, an Ultrogel AcA-34 column (310 × 8 mm) was used at 0.5 ml/min flow rate with the elution buffer that contained 1.5 mM EDTA, 150 mM NaCl, 0.02% sodium azide, and 50 mM Tris-HCl (pH 8.0). This buffer also contained sodium taurocholate in variable concentrations according to the total bile salt concentration previously determined in the low density fraction after ultracentrifugation. The excluded fractions, corresponding to non-micellar carriers of biliary lipids, were concentrated by ultrafiltration in Centricon-10 and then subjected to a second gel filtration on Sepharose CL-2B-300 (800 × 3 mm), equilibrated, and eluted in the same buffer as above but without bile salts. In this manner, we intended to preserve the proportion of lipid carriers of the low density fraction during the purification procedures.

To analyze the possibility of simple partition of biliary proteins between micelles and vesicles, we prepared, by ultracentrifugation, a vesicle-depleted native bile and we incubated this bile with artificial unilamellar vesicles (22) that had the same cholesterol and phospholipid composition as native biliary vesicles. After 24 h incubation at 37°C, we re-isolated the exogenous vesicles exactly as described for native bile.

Preparation of hepatic subcellular fractions

Rats were decapitated and livers were perfused in situ with ice-cold saline through the portal vein. The livers were removed and placed into cold 0.25 M sucrose, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, and 100 mM Tris-HCl (pH 7.4). All subsequent procedures were carried out at 0–4°C. The tissue was minced and homogenized with three passes in a Potter-Elvehjem tissue grinder (Arthur H. Thomas Company, Philadelphia, PA) equipped with a motor-driven Teflon pestle. Liver homogenates were fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and soluble (S) fractions, as described by de Duve et al. (23). A highly purified canalicular plasma membrane (24) was kindly provided by Dr. Luigi Accatino (Departamento de Gastroenterología, Pontificia Universidad Católica, Santiago, Chile).

Polyacrylamide gel electrophoresis

Proteins were precipitated from whole bile or from different bile–Metrizamide gradient fractions with trichloroacetic acid (7% w/v) and delipidated with cold ethanol–diethyl ether 1:3 prior to electrophoresis. The delipidated proteins were sedimented by low speed centrifugation, the organic solvent was discarded, and the protein pellet was dried under a N_2 stream. Then, the biliary protein pellets and the hepatic subcellular fractions were dissolved in sample buffer and electrophoresed on 9% polyacrylamide-SDS gels (SDS-PAGE) according to Laemmli (25). Gels were stained with Coomassie brilliant blue.

Purification of the 130-kDa protein associated to the non-micellar biliary lipid carriers and preparation of polyclonal antibodies

The 130-kDa protein antigen from purified lamellae was isolated by electroelution from polyacrylamide gel slices. Proteins associated to the non-micellar biliary lipid carriers were separated by preparative SDS-PAGE. The 130-kDa protein was located by Coomassie blue staining and then excised with a scalpel. The gel slice was transferred to a dialysis tube containing 192 mM glycine and 25 mM Tris-HCl (pH 7.4). The dialysis bag was placed in a horizontal chamber with 96 mM glycine and 12.5 mM Tris-HCl (pH 7.4). Electroelution was run at 50 mA overnight. Then the protein solution was removed from the dialysis tube, concentrated by ultrafiltration, and finally lyophilized on a Speed Vac. The purified 130-kDa protein antigen was emulsified with complete Freund's adjuvant and about 50 μg was injected in the popliteal lymph node of white female New Zealand rabbits. Intradermal booster injections of 100 μg antigen were given every week for three weeks. Blood samples were taken from the marginal ear vein to check the production of specific antibodies by microtiter and immunoblot. Immune serum was stored at −20°C.

Immunoblotting

For immunoblotting, SDS-PAGE-separated proteins from biliary and hepatic fractions were transferred overnight to nitrocellulose filters under a constant current (50 mA) at room temperature. The filters were blocked with 5% defatted milk in TBS (150 mM NaCl, 0.02% sodium azide, and 25 mM Tris-HCl, pH 7.4) and then incubated with the primary anti-130-kDa protein antibody, diluted 1/1000 in the same solution. The filters were washed (three times for 10 min) with TBS containing 0.1% Tween-20. Adsorbed IgGs were detected by incubating the filters with Protein A conjugated to alkaline phosphatase, diluted 1/1000. The alkaline phosphatase reaction was carried out with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates and 150 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 9.5) as reaction buffer. In some experiments, quantitative distribution of the 130-kDa protein was assessed by densitometric scanning of the immunoblotted membranes.

Immunohistochemistry

Fresh specimens of rat and human livers were fixed overnight by immersion in Bouin's fixative. Hepatic tissue was embedded in Paraplast. Four-μm sections were mounted onto glass slides and placed in a 60°C oven for 1 h. After dewaxing in xylene, tissue sections were rehydrated by passing through graded ethanol. Immunohistochemistry was performed with the peroxidase–anti-peroxidase method. The rehydrated sections were preincubated with methanol–H_2O_2 9:1 for 20 min to block nonspecific endogenous staining. Washing steps were carried out with peroxidase–anti-peroxidase buffer (130 mM NaCl, 3.5 mM KH_2PO_4, 10 mM Na_2PO_4, 3 mM sodium azide, and 40 mM Tris-HCl, pH 7.6) supplemented with 0.7% carrageenan when immunoreagents were used. First, the sections were incubated with the rabbit polyclonal anti-130-kDa protein antibody diluted 1/100. The samples were then incubated with goat anti-rabbit IgG antiserum at 1/20 dilution for 30 min and finally with rabbit peroxidase–anti-peroxidase complex (dilution 1/150) for 30 min. After washing, sections were developed in peroxidase–anti-peroxidase buffer plus 0.1% 3,3'-diaminobenzidine and 0.01% H_2O_2 for 10 min.

Electron microscopy

Fresh specimens obtained from bile–Metrizamide gradient fractions and from rat livers were fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2). Carbon films on mica substrates were floated on the fixed bile gradient fractions for 5 min, transferred to the staining solution for 3 min, picked up on 200-mesh copper grids, and air-dried on filter paper. The negative staining was performed with 2% aqueous uranyl acetate. One-mm^3 blocks of fixed rat liver were dehydrated in acetone and embedded in Epon. Thin sections were made using glass knives, mounted on copper grids, and stained with uranyl acetate and lead citrate. Sample observations were performed at 80 kV with a Siemens 102 electron microscope.

Amino acid sequencing of the 130-kDa lamellar protein

Proteins from purified non-micellar carriers of biliary cholesterol were electrophoresed as described above. Proteins were then electroblotted to polyvinylidene difluoride (PVDF) membranes in transfer buffer (10% methanol, 10 mM CAPS, pH 11.0). The 130-kDa protein was identified by Coomassie blue staining and the band was cut out with a clean razor. The protein was directly sequenced from the PVDF membrane on an Applied Biosystems sequenator equipped with on-line PTH analysis.

Chemical analysis

Cholesterol, phospholipids, and bile salts were quantitated as previously described (9). For phospholipid analysis, purified lamellae were extracted with chloroform–methanol 2:1 and the chloroform phase was dried under N_2. The extracted lipids were redissolved in n-hexane–2-propanol–water 6:8:1 and separated by thin-layer chromatography (TLC) on silica gel G plates developed in chloroform–methanol–acetic acid–water 50:30:8:4. Synthetic phospholipids were run in parallel as standards. Phospholipid classes were visualized by 8-aniline-naphthalenesulfonic acid and quantitated as above. Biliary cholesterol saturation index was calculated according to critical tables (26). Biliary proteins were measured by the method of Lowry et al. (27), including a precipitation/delipidation step for biliary proteins and proteins from bile–Metrizamide gradient fractions.

RESULTS

Effect of diosgenin plus simvastatin feeding on biliary lipid and protein composition

The first series of experiments was undertaken to produce a rat bile with a lipid composition similar to human native bile. This was achieved by feeding a 0.5% diosgenin plus 0.02% simvastatin diet. The diet was well tolerated by the animals. **Table 1** shows that after feeding the experimental diet for 1 week biliary cholesterol concentration increased more than 500% over the control value, whereas bile salt concentration was decreased by 50% and phospholipids remained in the normal range. These changes determined that bile samples from treated animals were highly supersaturated with cholesterol as compared with those from control rats. Phosphatidylcholine represented more than 90% of total biliary phos-

TABLE 1. Lipid and protein composition of bile from diosgenin plus simvastatin-fed rats and from human hepatic bile

Group	Biliary Lipid Concentration			Biliary Protein Concentration	Cholesterol Saturation
	Bile Salts	Phospholipids	Cholesterol		
	mM			*mg/ml*	%
Control rats (n=7)	43 ± 10	7.4 ± 1.3	0.8 ± 0.2	1.1 ± 0.1	39 ± 5
Diosgenin + simvastatin fed-rats (n=6)	23 ± 4*	7.0 ± 1.1	5.4 ± 0.7*	0.9 ± 0.1	283 ± 18*
Human (n=8)	21 ± 5	7.1 ± 0.8	4.9 ± 0.5	1.3 ± 0.3	265 ± 21

Rats were fed a control ground chow or ground chow containing 0.5% diosgenin plus 0.02% simvastatin for 7 days prior to the experiments. Phosphatidylcholines represented more than 90% of total biliary phospholipids, in both control and experimental rats, as determined by thin-layer chromatography. Values are the mean ± 1 SD. The asterisks indicate that the value is significantly different to controls at $P < 0.05$ level (unpaired Student's t test). The number of animals or patients in each group is shown in parentheses.

pholipids in both control and experimental rats (result not shown). In addition, biliary cholesterol, phospholipid, and bile salt concentrations of experimental rats were remarkably similar to those of human native bile. The cholesterol saturation index was 265 ± 21% and 283 ± 18% in human and experimental rat bile specimens, respectively. There was no significant difference in biliary protein concentration between control and experimental rats, compared with human bile. These results showed that diosgenin plus simvastatin feeding induces a biliary lipid composition pattern that was quite similar to native supersaturated human bile.

Lipid distribution, protein analysis, and morphological characterization of bile-Metrizamide gradients and liver tissue from diosgenin plus simvastatin-fed rats

Metrizamide gradient ultracentrifugation of bile from diosgenin plus simvastatin-fed rats produced an opalescent band in the top of the centrifuge tube at a fraction density similar to that of human bile. The concentrations of biliary lipids and protein in bile-Metrizamide gradients from experimental rats and human bile as a function of the density of the fractions are shown in **Table 2** and **Table 3,** respectively. There was a striking dissociation between the cholesterol and bile salt distributions in the gradients. Approximately 70% of total biliary cholesterol of diosgenin plus simvastatin-fed rats and of human bile specimens was concentrated in the low density fraction of the gradients ($d < 1.065$ g/ml), whereas in control rats only 10% of total biliary cholesterol was harvested in this fraction (result not shown). These low density fractions always presented a high cholesterol-phospholipid ratio (> 1.20). In contrast to cholesterol distribution, bile salts were concentrated in the higher density fraction of the bile-Metrizamide gradients.

Biliary protein concentration increased as a function of fraction densities. Although more than 80% of total biliary proteins was concentrated in the high density fractions, a minor but constant proportion (5-6%) of rat and human total biliary proteins migrated in the low density fraction. After column chromatography purification, only

TABLE 2. Biliary lipid and protein concentrations in bile-Metrizamide fractions from diosgenin plus simvastatin-fed rats

Fraction Number	Fraction Density	Biliary Lipid Concentration			Biliary Protein Concentration
		Bile Salts	Phospholipids	Cholesterol	
	g/ml		*mM*		*mg/ml*
1	1.055-1.065	12 ± 5	9.3 ± 4.0	11.7 ± 3.1	0.25 ± 0.1
2	1.065-1.079	20 ± 6	7.6 ± 5.0	7.1 ± 2.1	0.35 ± 0.2
3	1.078-1.094	23 ± 8	7.5 ± 3.2	1.9 ± 0.4	0.48 ± 0.2
4	1.094-1.110	25 ± 4	6.4 ± 3.2	0.8 ± 0.3	1.20 ± 0.3
5	1.110-1.129	31 ± 10	4.8 ± 0.8	0.7 ± 0.2	2.40 ± 0.2
	Whole bile	22 ± 6	7.1 ± 0.8	4.4 ± 0.4	0.98 ± 0.4

Values represent the mean ± 1 SD of five gradients. The total volumes of the gradients and of each fraction were approximately 6.0 and 1.2 ml, respectively. The lipid and protein recoveries from the gradients were always better than 80%.

TABLE 3. Biliary lipid and protein concentrations in bile-Metrizamide fractions from human hepatic bile

Fraction Number	Fraction Density	Biliary Lipid Concentration			Biliary Protein Concentration
		Bile Salts	Phospholipid	Cholesterol	
	g/ml		*mM*		*mg/ml*
1	1.051-1.063	15 ± 3	8.5 ± 3.1	10.9 ± 2.5	0.31 ± 0.2
2	1.063-1.075	18 ± 4	8.4 ± 3.2	9.4 ± 3.0	0.38 ± 0.1
3	1.075-1.095	20 ± 5	8.0 ± 2.8	2.3 ± 0.5	0.54 ± 0.2
4	1.095-1.112	17 ± 3	7.1 ± 3.5	0.8 ± 0.4	1.56 ± 0.2
5	1.112-1.133	35 ± 6	5.0 ± 2.1	0.6 ± 0.4	2.71 ± 0.3
	Whole bile	21 ± 3	7.3 ± 0.5	4.8 ± 0.6	1.1 ± 0.4

Values represent the mean ± 1 SD of four gradients. The total volumes of the gradients and of each fractions are the same as rat bile-Metrizamide gradients. The lipid and protein recoveries were at least 87%.

30-40% of the protein remained associated to the non-micellar carriers in both species.

Fig. 1 shows the morphological analysis of the low density fraction of bile-Metrizamide gradients from both human and rat bile. Electron microscopy demonstrated a great abundance of multilamellar structures in the form of stacked discs and variable coiled myelin-like sheets in both specimens with a minor proportion of unilamellar vesicles. The interlamellar distance was approximately 3-5 nm.

Thin sections of liver specimens from diosgenin plus simvastatin-fed rats were also processed for electron microscopy. Low magnification of the liver sections (**Fig. 2**) showed the presence of multilamellar bodies and numerous unilamellar vesicle structures within the bile canaliculi. Higher magnification indicated that the lamel-

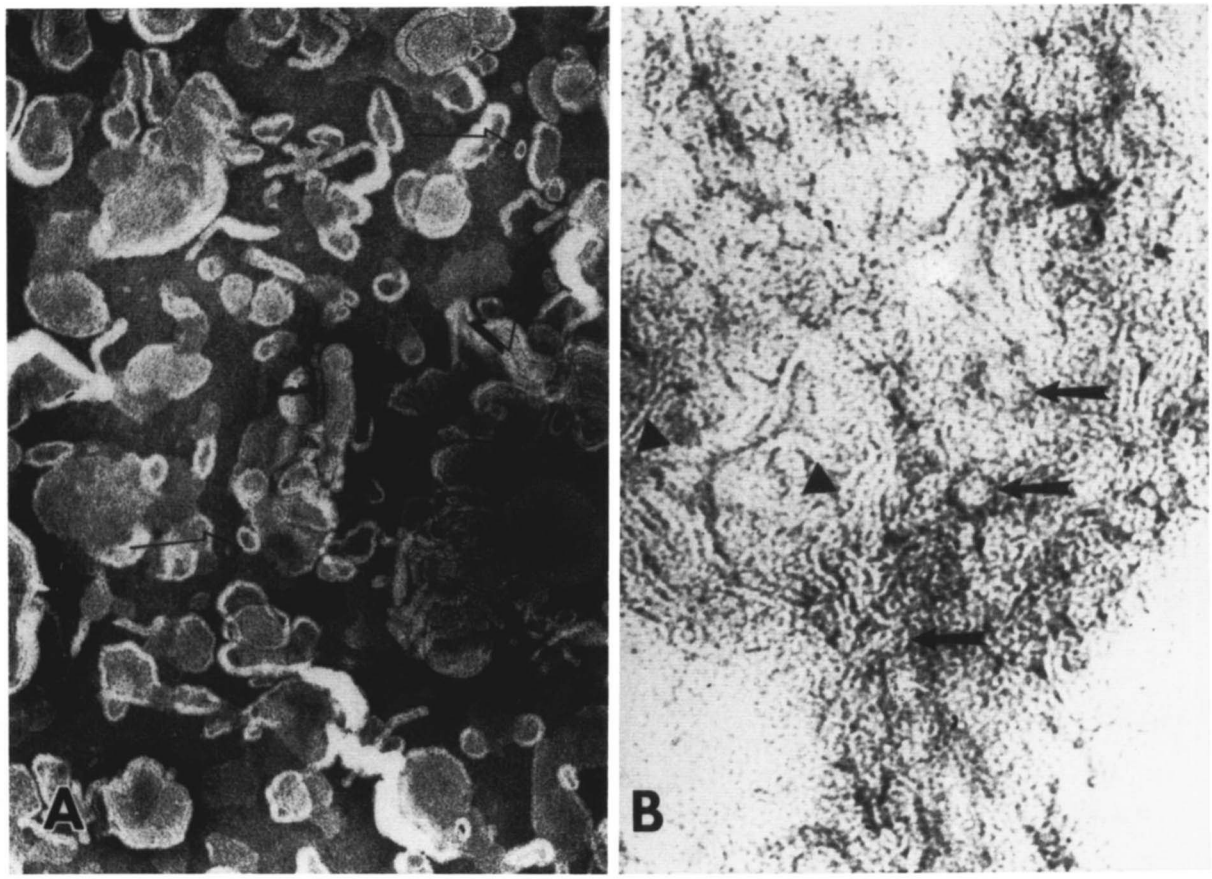

Fig. 1. Electron micrograph of rat (A) and human (B) low density fraction of a bile-Metrizamide gradient. Partly collapsed microvesicles (arrows) and intermingled multilayered lamellar structures (arrowheads) are observed. Uranyl acetate and lead citrate staining. Magnification: (A), 80,000 × and (B), 160,000 ×.

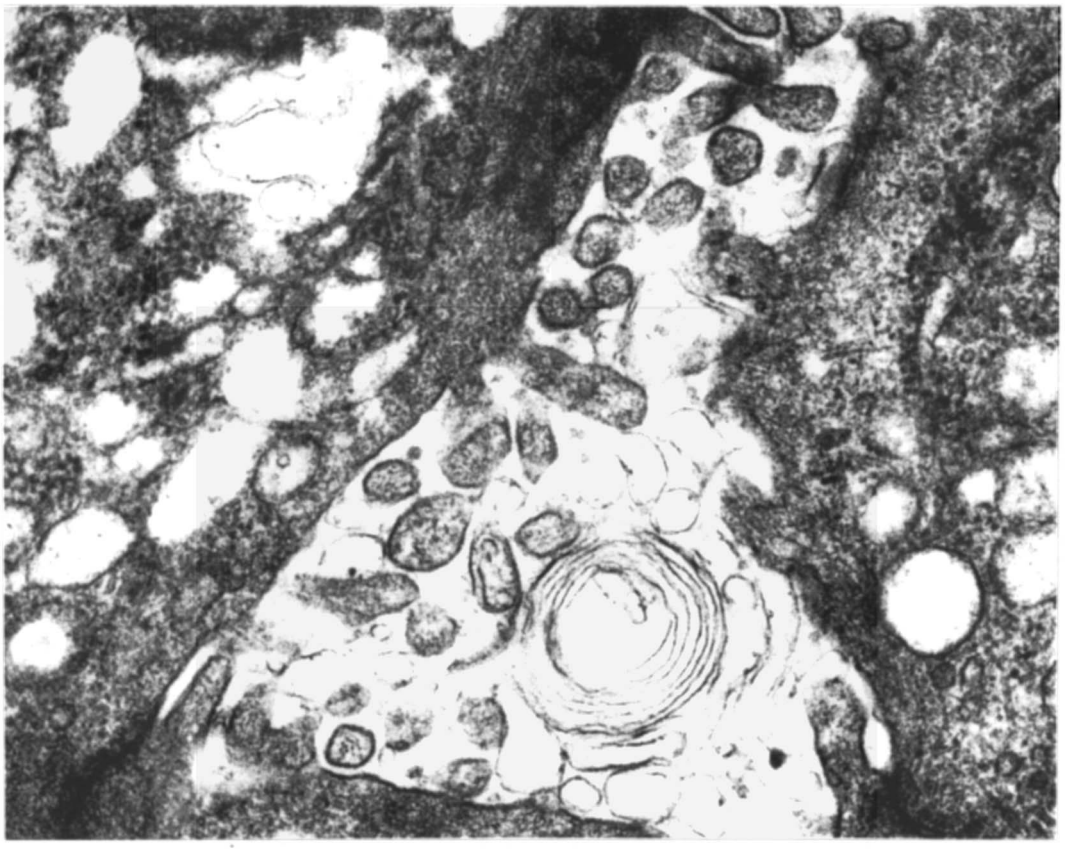

Fig. 2. Electron micrograph of a liver section from diosgenin plus simvastatin-fed rats. A bile canaliculus with a multilamellar body is shown. Some uncoiled lamellae are seen near the multilamellar body. Numerous unilamellar vesicles can be identified within the canalicular lumen, especially in the upper part of the micrograph. Also note some pericanalicular multivesicular bodies. Uranyl acetate and lead citrate staining. Magnification: 35,600 ×.

lar structures exhibited bilayer staining properties. The pericanalicular cytoplasm showed some multivesicular bodies, but not multilamellar structures similar to those found in canaliculi.

To further analyze the homology between these particulate structures from rat and human bile and to examine whether a specific protein subset was separated after gradient ultracentrifugation and chromatographic purification, the protein composition of isolated and purified biliary lamellar complexes in both species was assessed by non-reducing SDS-PAGE, as shown in **Fig. 3.** Whole rat bile (lane 1) showed a similar, but not identical, protein pattern as compared with whole human bile (lane 4), where several proteins were resolved. As expected from protein determination, in both species the highest density fraction of the bile–Metrizamide gradients contained the major proportion of total biliary proteins (lane 2 and lane 5). As shown in lane 3, the low density lamellar particles isolated and purified from diosgenin plus simvastatin-fed rats had a characteristic protein profile that was clearly distinct from that of the high density fractions. The lamellar protein pattern was extremely constant in five different pooled rat bile samples and it was remarkably similar to biliary lamellae isolated and purified from human native bile (lane 6). In both species, it was possible to resolve at least five proteins with apparent molecular mass of 130, 114, 86, 68, and 52 kDa. The relative distribution of the lamellar proteins when compared with whole native bile

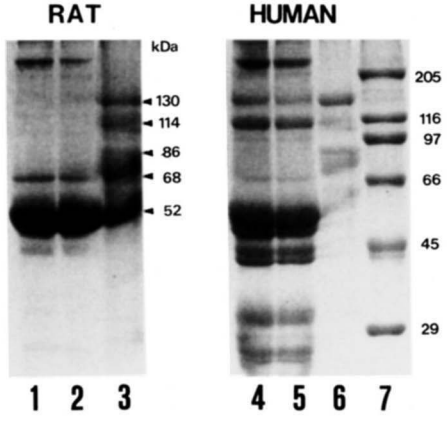

Fig. 3. Protein composition analysis by SDS-PAGE of fractions from bile–Metrizamide gradients. Lanes 1 and 4 correspond to whole native bile; lanes 2 and 5 correspond to the high density fraction (d 1.110–1.133 g/ml); lanes 3 and 6 correspond to the low density fractions (d 1.051–1.065 g/ml) after purification by two gel filtration chromatographies. Lane 7 shows the molecular weight standards. The arrow heads indicate the molecular weights of lamellar-associated proteins. One hundred μg of protein was loaded in each lane. Gels were stained with Coomassie blue.

showed that the 130-, 114-, 86-, and 68-kDa proteins were highly enriched in the lamellar fraction, unlike the 52-kDa polypeptide (previously identified as albumin (18)) and other minor proteins that were clearly decreased in the purified low density fraction of bile–Metrizamide gradients. Taken together, the data suggested that lamellar complexes are the major carrier of biliary cholesterol in diosgenin plus simvastatin-fed rats and showed a protein composition and morphological structure quite similar to those found in human bile.

Distribution of biliary lamellae-associated 130-kDa protein in bile–Metrizamide gradients and in hepatic subcellular fractions from diosgenin plus simvastatin-fed rats

The 130-kDa protein from human biliary lamellar particles was electroeluted from SDS-PAGE and used as antigen for rabbit polyclonal antibody production. The immune serum obtained recognized the human 130-kDa antigen and it also cross-reacted with the 130-kDa protein from rat biliary lamellar carriers. The pre-immune serum gave negative results for both antigens (data not shown). This anti-130-kDa protein polyclonal antibody was used for immunoblotting and for immunohistochemistry.

From SDS-PAGE, we thought that if the 130-kDa protein was a marker protein for biliary lamellae, a significant proportion of total 130-kDa protein should be associated with the purified bile–Metrizamide fractions as compared with the high density fractions of the gradients. As shown in **Fig. 4A,** by immunoblotting of human bile–Metrizamide gradients about 50–70% of the total biliary 130-kDa protein was demonstrated to be in the non-purified lamellar fraction (lane 6) and it was highly enriched in the purified lamellar fraction (lane 7). This result clearly contrasted with albumin (Fig. 4B), the major biliary protein, which is preferentially concentrated in the high density fractions (lanes 1–3) and it was detected in very low amount in purified lamellae (lane 7).

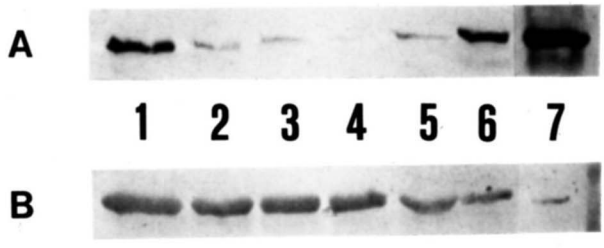

Fig. 4. Distribution analysis by Western blotting of the 130-kDa lamellar protein (A) and albumin (B) in a human bile–Metrizamide density gradient. Lane 1 indicates the high density fraction; lane 6, the low density fraction containing lamellae; lanes 2 to 5, correspond to the intermediate density fractions. Lane 7 is a purified lamellar fraction by two sequential gel filtration chromatographies (Ultrogel AcA-34 and Sepharose CL-2B-300). The same volumes (50 μl) of each fraction were precipitated and delipidated prior to electrophoresis.

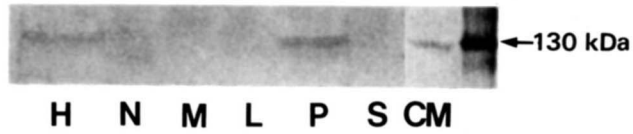

Fig. 5. Hepatic subcellular distribution of the 130-kDa lamellar protein immunodetected by Western blotting. Total rat liver homogenate (H) was fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), soluble (S), and purified canalicular plasma membrane (CM) fractions. The left lane corresponds to isolated and purified biliary lamellae as a positive control of the immunoblot. One hundred μg of protein was loaded in each lane.

Because the 130-kDa protein is an amphipatic biliary glycoprotein (19), it was thought that this protein might be associated to biliary vesicles only by simple partitioning into the more hydrophobic lamellar environment of the biliary vesicles. To evaluate this possibility, we prepared a vesicle-depleted native bile by ultracentrifugation and we incubated this bile with artificial unilamellar vesicles with the same cholesterol and phospholipid composition as native biliary vesicles. After the incubation, we re-isolated the artificial vesicles and analyzed the distribution of the remaining biliary 130-kDa protein in a bile–Metrizamide gradient as it was performed for native bile. The re-isolated artificial vesicles contained only a minor proportion of the remaining 130-kDa biliary protein (less than 8%). Taken together, these data strongly suggested that the 130-kDa protein is significantly associated with native biliary vesicles and multilamellae in this experimental model.

To examine the hepatic origin and distribution of the 130-kDa lamellar protein, we analyzed its association with isolated subcellular fractions by immunoblotting. As shown in **Fig. 5,** the 130-kDa protein was clearly detected in total hepatic homogenate (lane H) and it was shown only in a total microsomal fraction (lane P), which contains plasma membrane, and in a subcellular fraction enriched in canalicular plasma membrane (lane CM). The 130-kDa protein was not detected in sinusoidal plasma membrane (data not shown).

Localization of the biliary 130-kDa protein by hepatic immunohistochemistry

In the next series of experiments, the polyclonal antibody against the 130-kDa protein was used for immunohistochemistry studies both in rat and human liver sections. The immunohistochemical localization of the 130-kDa protein is presented in **Fig. 6.** The histochemical pattern was highly distinctive of the biliary canalicular domain: anastomosing networks of branched channels as well as circular profiles restricted to regions between adjacent cells. At high magnification, labeling appears to be associated either with the luminal cell surface or with intraluminal convoluted particles. The liver staining pattern

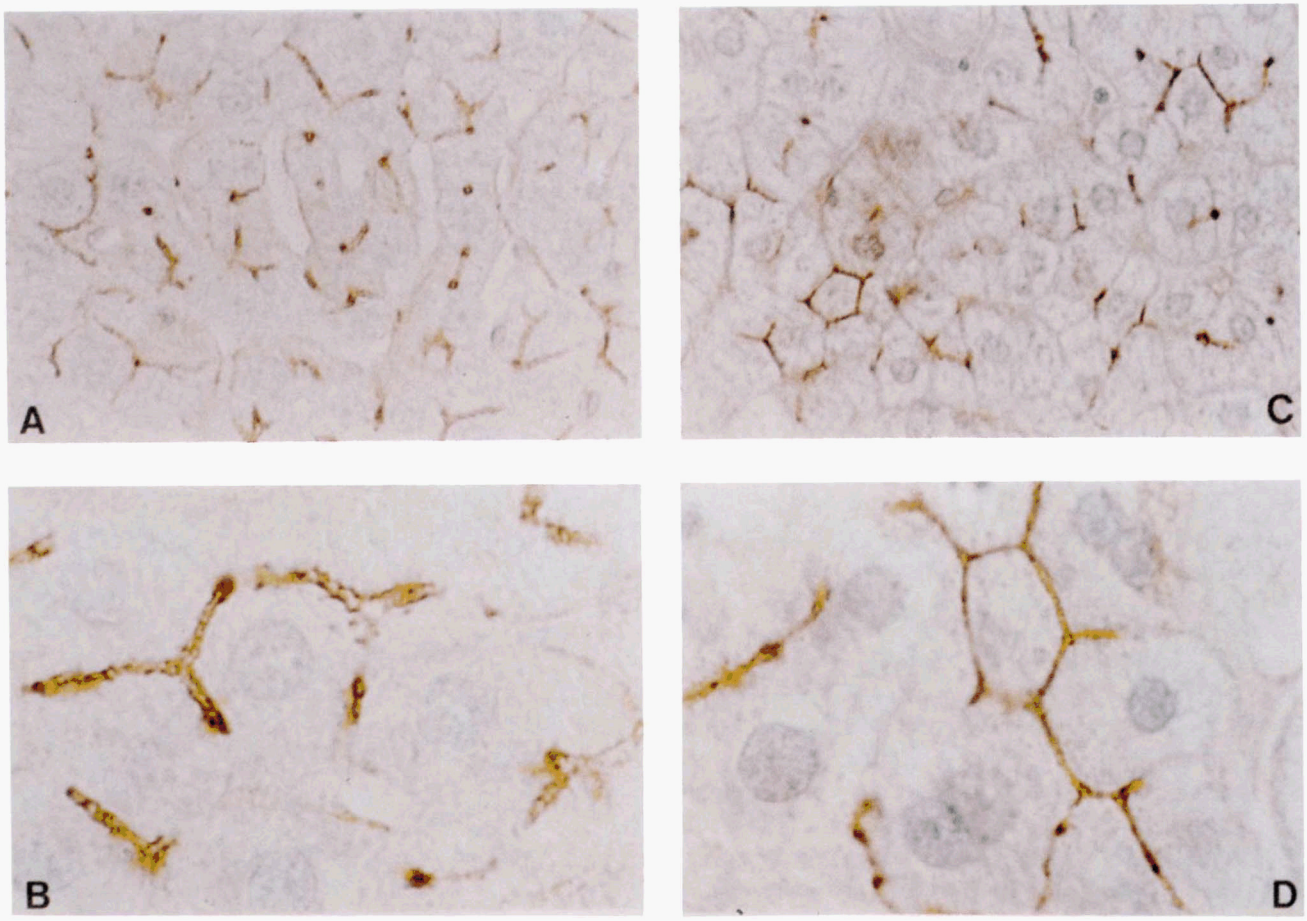

Fig. 6. Histological localization of the 130-kDa lamellar protein by immunocytochemistry. Rat (A, B) and human (C, D) liver sections were analyzed by the peroxidase–anti-peroxidase method. Magnification: 40 × (A, C) and 100 × (B, D).

was similar in both species. No sinusoidal plasma membrane or intracellular staining was observed and controls done with preimmune serum were blank.

Identification of the 130-kDa protein by amino acid sequencing and immunoblotting

Finally, the 130-kDa human lamellar protein was identified by amino acid sequencing of the amino terminus of the biliary-secreted and isolated protein. The analysis revealed the sequence AKGFYISKSLGILGILL. By an amino acid sequence data bank search, a complete identity was found between the 130-kDa lamellar protein sequence and the cytoplasmic NH_2 terminal domain of the membrane-bound protein aminopeptidase N (28). To further confirm this finding, a positive reaction in immunoblot was demonstrated by using commercially available purified aminopeptidase N as antigen and the anti-130-kDa protein polyclonal immune serum as primary antibody (result not shown). The identification of the 130-kDa protein as aminopeptidase N is consistent with the immunological studies, which determined its hepatic distribution and localization to the canalicular plasma membrane domain.

DISCUSSION

The present study demonstrates that diosgenin plus simvastatin feeding induces the hepatic secretion of a human-like highly cholesterol-supersaturated bile in the rat. This experimental manipulation determines that the major proportion of biliary cholesterol is transported in lamellar carriers (unilamellar vesicles and multilamellae), as also occurs in human native bile. A remarkable finding was the morphological evidence of intracanalicular multilamellar bodies and unilamellar vesicles. A distinct, constant, and similar protein profile was associated with lamellar carriers in both rat and human bile. The hepatic localization and distribution of the 130-kDa lamellar protein, identified as aminopeptidase N, reveal a distinctive pattern of the canalicular plasma membrane domain.

In bile, the relative cholesterol content can be modulated by a great variety of experimental procedures (29–33). Experimentally induced changes in biliary

cholesterol secretion are of particular interest because they allow the identification of the mechanisms involved in biliary cholesterol supersaturation. In the present experimental model, the simultaneous administration of diosgenin plus simvastatin to the rat induces the hepatic secretion of a highly cholesterol-supersaturated bile, with a lipid composition identical to native hepatic bile from patients with cholesterol gallstone disease. The addition of simvastatin significantly increased the previously described biliary cholesterol hypersecretion determined by diosgenin feeding (30, 32). The effect of this pharmacological manipulation is determined by a massive increment in biliary cholesterol secretion with minor changes in phospholipid and bile salt output (results not shown).

The functional role of unilamellar vesicles as a native biliary cholesterol carrier has been clearly established (3–9), including the present morphological data. The existence of biliary multilamellar structures have been previously described in both artificial and native bile, but they were considered as micellar aggregates, lipoprotein complexes, or artifacts (34–36). Recently, it has been shown by both small-angle X-ray diffraction and negative staining electron microscopy that phospholipid lamellae are important carriers of biliary cholesterol in human bile (10). In the present study, we used density gradient ultracentrifugation to separate biliary lipid carriers from diosgenin plus simvastatin-fed rats. About 70% of biliary cholesterol is found in the low density fraction of the gradients and electron microscopy of these fractions showed multilamellae and unilamellar vesicles. Moreover, the observation of multilamellar bodies within the canaliculi is the first visual demonstration of intrahepatic multilamellar carriers of biliary lipids. This finding is an important morphological evidence to support the hypothesis that biliary cholesterol is preferentially transported and primarily secreted in lamellar carriers. We think that the secretion of cholesterol supersaturated bile allowed us to detect rat multilamellae, which are probably solubilized into mixed micelles at the high relative bile salt concentration normally found in unsaturated rat bile. Phospholipid secretion is doubled in the diosgenin–simvastatin-fed rats (results not shown), suggesting that the process of biliary lamellar production is increased in this model. The high cholesterol–phospholipid ratio probably increases the resistance of the lamellae to bile salt dissolution, thus making possible their detection by transmission electron microscopy. However, electron microscopy of lipid-rich protein-poor particles, such as biliary vesicles, can perturb the native ultrastructure during sample preparation for negative staining. Therefore, it is not possible to rule out that the morphological appearance of the negative stained multilamellae was determined by dehydration and heavy metal exposure. But using more conventional electron microscopy (fixation, embedding, thin sectioning, and positive staining) of hepatic tissue, we were also able to show multilamellae within the canaliculi and one can reasonably consider that multilamellae are not artefactually induced by sample processing. In addition, phospholipid lamellae have been clearly demonstrated in bile by a nondisturbing independent physical method, such as small-angle X-ray diffraction (10).

A small but constant fraction of biliary proteins was consistently associated with isolated and purified biliary lamellae in this study, as it has been previously reported (18, 19). Recent studies from this laboratory have demonstrated that the 130-, 114-, and 68-kDa lamellar proteins are hydrophobic glycoproteins (19). Protein composition analysis on SDS-PAGE demonstrated that the lamellar material from both rat and human bile contains, after their chromatographic purification, a distinctive and constant protein profile that was preferentially associated with the purified lamellar fraction of the gradients. It is interesting to note that the secretory multilamellar bodies shown in the liver of the diosgenin plus simvastatin-fed rats have an ultrastructural appearance similar to lamellar bodies described in other epithelia (37), such as lung alveoli, gastrointestinal mucosa, tongue papillae, and skin. A comparison of the biliary lamellar protein pattern with those already described in lamellar complexes from other tissues, especially with the small intestinal surfactant-like material (38), reveals a number of potentially common protein components, but adequate identification is still needed. For example, small intestinal surfactant-like material is enriched with alkaline phosphatase and also contains aminopeptidase-N (39), which are membrane-bound proteins. In addition, SDS-PAGE of this intestinal material presents a protein profile with three polypeptides between 66 and 130 kDa (39), that are in the molecular weight range of the biliary lamellar proteins identified in this study. Further protein characterization and careful comparisons between lamellar complexes from different sources might detect related proteins, which should eventually help in the understanding of the nature and origin of these widely distributed lamellar particles.

The intracellular mechanisms of biliary lipid secretion from the liver are still unknown (9, 12, 14). Although unilamellar vesicles had been previously detected in rat liver (9) and the present study identified multilamellar bodies within the bile canaliculi, it has not been elucidated whether these cholesterol and phospholipid lamellar structures originate inside the hepatocyte, at the canalicular membrane, or within the canalicular lumen. In this order, is interesting to note the association of aminopeptidase N to native biliary lamellae evidenced in the present study. The amino acid sequencing of the biliary secreted lamellar aminopeptidase N revealed that this enzyme is present in the bile as a whole protein, including its transmembrane and cytoplasmic domain (28). This finding is consistent with the previous evidence that most of the aminopeptidase N present in bile is an amphiphilic form (19, 40). The present experiments of biliary aminopeptidase N interaction with artificial unilamellar vesicles do

not allow us to postulate a simple random partitioning effect of this hydrophobic biliary protein to explain the association of aminopeptidase N with native biliary lamellae after separate secretion of each of them into bile. The present results more likely suggest that the canalicular membrane might be shed during the biliary lipid secretory process, including some transmembrane proteins such as aminopeptidase N.

Plasma membrane shedding is a widely distributed biological phenomenon, whereby normal eukaryotic cells release some fragments of the plasma membrane and sometimes specific cytoplasmic material into the extracellular space (41, 42). This process can take a variety of forms involving relatively random large membrane sheets or only very small and specific portions of plasma membrane. We think that the secretion of biliary lamellae as a process of random canalicular membrane shedding is very unlikely, because such lamellar shedding has been rarely observed. In addition, there are significant differences in lipid and protein composition between biliary lamellae and the whole canalicular membrane, such as increased cholesterol–phospholipid ratio, percent contribution of phosphatidylcholine to total phospholipids, saturated fatty acid chains, total lipid–protein ratio, and different total protein composition of biliary lamellar complexes (present study) as compared with canalicular plasma membrane (24, 43). This random shedding, bile salt-dependent process, may not explain, as a unique mechanism, the total lipid mass secreted into the bile, as bile salt-dependent lipid solubilization from different membranes in vitro is apparently very slow. The present findings, however, are more consistent with the hypothesis that selected domains of the canalicular membrane are shed by a specific topologically restricted budding process, contributing in part to biliary lipid secretion (11, 12, 14, 44). Canalicular membrane microdomains composed of biliary-type lipids and selectively segregated membrane-bound proteins (as aminopeptidase N) could be pinched off in a vesiculated form into the canalicular lumen, wherein the unilamellar vesicles by interconversion yield secondary structures as multilamellae and mixed micelles. It is of interest that this postulated model resembles the budding of viruses from the cell surface, in which viral envelope transmembrane proteins are highly enriched in budding microdomains with host cell lipids and exclusion of host membrane proteins (45). After secretion, the biliary lamellar particles could remain as proteoliposome structures within the canaliculus and they would mix with vesicular formations derived into the bile by other hepatocellular mechanisms that would be responsible for the secretion of the major proportion of biliary lipids. In this order, exocytosis represents a more efficient and selective secretory process consistent with the high amount and the specificity of the lipid species secreted into the bile (12, 14). In fact, pericanalicular vesicles (46) and multivesicular bodies (47) have been reported within the hepatocyte and they might correspond to intermediate organelles in the exocytotic secretory pathway of biliary lipids.

We think that the present model of cholesterol lamellar secretion of supersaturated bile may be a useful tool for the experimental approach to the study of biliary lipid secretion. The isolation, characterization, and localization of the lamellar carriers of biliary lipids can be considered as a first step in the elucidation of the cellular mechanisms involved in biliary lipid secretion. Using this model, further experiments could provide a better understanding and new insights into the cell and molecular biology of biliary lipid secretion, both in physiological and in pathological conditions, such as biliary cholesterol precipitation and gallstone formation.

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