Structural and Functional Polarity of Canalicular and Basolateral Plasma Membrane Vesicles Isolated in High Yield from Rat Liver

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ABSTRACT A method has been developed for routine high yield separation of canalicular (cLPM) from basolateral (blLPM) liver plasma membrane vesicles of rat liver. Using a combination of rate zonal floatation (TZ-28 zonal rotor, Sorvall) and high speed centrifugation through discontinuous sucrose gradients, 9–16 mg of cLPM and 15–28 mg of blLPM protein can be isolated in 1 d. cLPM are free of the basolateral markers Na^+/K^+-ATPase and glucagon-stimulatable adenylate cyclase activities, but are highly enriched with respect to homogenate in the "canalicular marker" enzyme activities leucylnaphthylamidase (48-fold), γ-glutamyltranspeptidase (60-fold), 5′-nucleotidase (64-fold), alkaline phosphatase (71-fold), Mg^{++}-ATPase (83-fold), and alkaline phosphodiesterase I (116-fold). In contrast, blLPM are 34-fold enriched in Na^+/K^+-ATPase activity, exhibit considerable glucagon-stimulatable adenylate cyclase activity, and demonstrate a 4- to 15-fold increase over homogenate in the various "canalicular markers." cLPM have a twofold higher content of sialic acids, cholesterol, and sphingomyelin compared with blLPM. At least three canalicular- (130,000, 100,000, and 58,000 mol wt) and several basolateral-specific protein bands have been detected after SDS PAGE of the two LPM subfractions. Specifically, the immunoglobin A–binding secretory component is restricted to blLPM as demonstrated by immunochemical techniques. These data indicate virtually complete separation of basolateral from canalicular LPM and demonstrate multiple functional and compositional polarity between the two surface domains of hepatocytes.

Hepatocytes like other secretory cells represent highly polarized units containing three morphologically and functionally distinct surface domains (1, 2). The sinusoidal front, characterized by numerous microvilli extending into the space of Disse, provides for efficient exchange of various ions, organic solutes, and proteins with the blood (3, 4). The lateral surface, in contact with neighboring hepatocytes, is specialized for cell adhesion and intracellular communications and is characterized by tight junctions, desmosomes, and gap junctions (5). Since both the sinusoidal front and the lateral surface (i.e., the "basolateral" pole of hepatocytes) are in physical continuity and directly exposed to sinusoidal blood plasma, these two plasma membrane domains are thought to be functionally equivalent (1, 6). In contrast, the bile canalicular or apical pole of the cell, which accounts for only 13% of the plasma membrane surface area, is separated from the plasma space by tight junctional complexes. These tight junctions encircle the hepatocyte like a belt or girdle (2). They exhibit only limited permeability to small plasma solutes and effectively prevent the regurgitation of bile components into blood. Within the minute channels of bile canaliculi, the canalicular membranes are folded into microvilli and are highly specialized for the primary secretion of bile (7), but the molecular mechanisms or driving forces for canalicular bile formation are still unknown. Progress in understanding the functional properties of this apical pole of the hepatocyte is dependent in large part on the ability to purify canalicular liver plasma membranes (cLPM)[1] in a sufficient yield to permit extensive

[1] *Abbreviations used in this paper:* blLPM, isolated basolateral LPM vesicles; cAMP, cyclic AMP; cLPM, isolated canalicular LPM vesicles; IgA, immunoglobin A; LPM, liver plasma membranes; NC, nitrocellulose; RE, relative enrichment of enzyme activities over homogenate; SC, secretory component.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 MARCH 1984 991–1000
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functional studies.

There is increasing evidence that there are morphological and functional differences between the basolateral and canalicular surface domains of the hepatocyte. For example, the asialoglycoprotein receptor (8), a variety of hormone receptors (1, 9), the glucagon-stimulatable adenylate cyclase (10), and the integral membrane pump Na^+K^+-ATPase (11, 12) are restricted to the basolateral plasma membrane. While a similar surface distribution is suggested for the receptor of polymeric immunoglobin A (IgA), the secretory component (SC), the association of SC with the cLPM is still controversial (13–15). Furthermore, although several marker enzyme activities such as Mg^+-ATPase, alkaline phosphatase, alkaline phosphodiesterase I, leucylnaphthylamidase, and γ-glutamyltranspeptidase are highly enriched at the canalicular front (10, 16), it has not been established that these putative canalicular markers are exclusively localized to this plasma membrane domain. Therefore, the selective canalicular origin of an isolated liver plasma membrane (LPM) subfraction can only be proven by the simultaneous demonstration of the absence of typical basolateral membrane components.

Current procedures for the isolation and purification of cLPM are either cumbersome, requiring specialized zonal rotors and lengthy ultracentrifugation steps (16, 17), or they result in a low total yield making it difficult to carry out functional studies (18, 19). Furthermore, all of the reported cLPM subfractions are also enriched in Na^+K^+-ATPase activity indicating significant contamination with basolateral membrane fragments. Thus, the purification and characterization of cLPM in high yield remains an important goal to elucidate transcanalicular solute transport processes. In the present study we describe a new subcellular fractionation procedure that permits the simultaneous isolation of an average of 13 mg protein of highly purified cLPM and 22 mg protein of basolateral plasma membranes (blLPM) in vesicular form from 110 g of rat liver. Extensive characterization of these two LPM subfractions demonstrates considerable structural and functional polarity of the hepatocyte with respect to membrane lipids, proteins, and various enzyme activities. In addition, the isolated LPM subfractions were used to exclusively localize SC in blLPM indicating that this IgA receptor is not an integral component of the bile canalicular membrane. Part of this work has been presented in preliminary form (20).

MATERIALS AND METHODS

Materials

The enzyme substrates ATP, AMP, NADPH (type I), NADH (disodium salt, grade III), succinate (disodium salt), *p*-nitrophenylphosphate (disodium salt), L-leucyl-β-naphthylamide, γ-glutamyl-*p*-nitroanilide, *p*-nitrophenyl thymidine-5′-phosphate, uridine-5′-diphosphogalactose, and *N*-acetylglucosamine were obtained from Sigma Chemical Co., St. Louis, MO. Kynuramine dihydrobromide was from Calbiochem-Behring Corp., La Jolla, CA. All other chemical and reagents (analytical grade) were purchased either from Sigma Chemical Co.; P-L Biochemicals, Inc., Milwaukee, WI.; J. T. Baker Chemical Co., Phillipsburg, NJ; Eastman Kodak Co., Rochester, N.Y.; Mallinckrodt, St. Louis, MO; or Bio-Rad Laboratories, Richmond, CA. Phospholipid standards were from Supelco, Inc., Bellefonte, PA. [α-^{32}P]ATP was obtained from Amersham, Arlington Heights, IL. [G-^{3}H]AMP and [galactose-U-^{14}C]UDP galactose were from New England Nuclear, Boston, MA.

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing 200–250 g were used throughout this study. The animals had free access to water, were fed Purina Rodent Chow ad libitum, and were housed in a constant temperature-humidity environment with alternating 12-h light (5 AM to 5 PM) and dark cycles. Fed animals were regularly killed by decapitation between 7:30 and 8:30 AM.

Isolation of LPM

All solutions, prepared 24–48 h before use, were stored at 4°C. Densities of the sucrose solutions were adjusted at room temperature by refractometry. All isolation steps were done at 0–4°C. Routinely, 10–12 fed animals (200–240 g) were killed and their livers (100–110 g) were rapidly removed and chilled on ice. 10-g portions of liver were cut into small pieces, washed three times in 80 ml cold 1 mM $NaHCO_3$ (pH 7.4), and homogenized in the same volume of 1 mM $NaHCO_3$ with a loose (Type A) Dounce homogenizer (seven up-and-down strokes). The homogenate was further diluted to 1800–2000 ml with cold $NaHCO_3$ and filtered twice through two layers of 60-grade cheesecloth. Centrifugation at 1,500 *g* for 15 min (GSA rotor, E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT) gave a "crude nuclear pellet" which was resuspended in 5.5 vol of 56% sucrose (wt/wt; density = 1.2623) and stirred for 15 min to disrupt membrane aggregates. The sample was then dynamically loaded onto a 100-ml cushion of 56% (wt/wt) sucrose with a variable speed Sorvall pump into the zonal rotor TZ-28 (Sorvall Instruments) and overlayed by 400 ml of 44% sucrose (wt/wt; density = 1.1972) and 200 ml of 36.5% sucrose (wt/wt; density = 1.1587), respectively. Finally, the rotor was filled to its total volume capacity (1,350 ml) with 0.25 M sucrose. During the entire loading procedure the rotor was running at 3,000 rpm. The completed discontinuous sucrose gradient system was centrifuged at 20,000 rpm for 120 min. After slow deceleration to a complete stop, 70 15-ml fractions were collected from the bottom of the rotor and analyzed for density, turbidity (absorbance at 700 nm), and marker enzyme activities. Fractions containing the bulk of plasma membrane fragments (e.g., fraction 54–64 out of 70 in Fig. 1) were combined and diluted with $NaHCO_3$ to 1,000 ml. The suspension was centrifuged at 7,500 *g* for 15 min, the pellet was gently resuspended (by vortex mixing) in 250 ml $NaHCO_3$, and the material representing the "mixed LPM" was resedimented at 2,700 *g* for 15 min.

Although no complete separation of basolateral from canalicular LPM has been so far reported, several previous studies suggested that canalicular LPM have a lower density than basolateral LPM (e.g., references 10 and 16–18). Therefore, extensive fragmentation of "mixed LPM" followed by high speed sucrose gradient centrifugation was tried to reduce reported cross-contaminations between the two LPM subfractions. Thus, mixed LPM were diluted with 0.25 M sucrose to a total volume of 25 ml and homogenized with a tight Type B glass-glass Dounce homogenizer by 50 up-and-down strokes. 3.5 ml of mixed LPM was layered on top of a three-step sucrose gradient consisting of 4 ml 38% (wt/wt; density = 1.1663), 2.5 ml 34% (wt/wt; density = 1.1463), and 2.5 ml 31% (wt/wt; density = 1.1318) sucrose. The tubes were centrifuged at 40,000 rpm (195,700 g_{ave}) for 3 h in the Beckman SW 41-rotor (Beckman Instruments Inc., Palo Alto, CA). This resulted in three distinctive bands and a pellet. Based on the morphological, enzymatic, and chemical characteristics, the band recovered from the top of the 31% sucrose layer will be designated cLPM whereas the material at the 34/38% interface represent the blLPM. All membranes contained within the 31 and 34% sucrose down to the upper edge of the blLPM band were collected together and are referred to as 34% membranes. All membranes contained within the 38% sucrose and in the pellet are referred to as pellet. The LPM subfractions were diluted in 0.25 M sucrose, sedimented at 105,000 *g* for 60 min, and finally resuspended in 0.25 M sucrose-0.2 mM $CaCl_2$-10 mM HEPES/Tris, pH 7.5, except for those used for the determinations of adenylate cyclase activity and phospholipids where EDTA (1 mM) was added.

Biological Methods

ENZYME ASSAYS: All enzyme activities except galactosyltransferase were routinely measured on fractions stored at 4°C for up to 12 h. Galactosyltransferase was measured on samples kept frozen at −20°C for up to 1 mo. To verify that no enzyme activities were lost during the storage time, occasional measurements on fresh membrane preparations were also done. All frozen samples were quick-thawed immediately before use by immersion into 37°C warm water.

5′-nucleotidase activity was measured by the radioactive assay using tritiated 5′AMP as substrate (21). The Mg^+-ATPase and the ouabain-sensitive Na^+K^+-ATPase activities were determined by the coupled kinetic assay as modified by Scharschmidt et al. (22). Alkaline phosphatase was assayed as described (23) using *p*-nitrophenylphosphate as substrate. The method of Goldbarg and Rutenburg (24) was used for determination of the leucyl-β-naphthylamidase activity. γ-Glutamyltranspeptidase and alkaline phosphodiesterase I activities

were measured according to Orlowski and Meister (25) and Razzel (26), respectively. To test for the presence of intracellular organelles, we measured the following marker enzyme activities: NADH and succinate cytochrome *c* reductase, cytochrome *c* oxidase and monoaminooxidase for mitochondria (27, 28); NADPH cytochrome *c* reductase for microsomes (27); acid phosphatase for lysosomes (29); and galactosyltransferase for Golgi membranes (30).

The adenylate cyclase activity was determined by an adaptation of a previously described method (31). The reaction mixture contained 100,000 cpm of [^{32}P]ATP, 50 mM Tris-HCl at pH 7.4, 5.0 mM $MgCl_2$, 10 mM KCl, 1.0 mM EGTA, BSA at 1 mg/ml, creatine kinase at 0.11 U/ml, 5 mM creatine phosphate, 0.125 mM ATP, 1.0 mM cyclic AMP (cAMP), 2.0 mM dithiothreitol, and 10 μM 5′-guanylylimidodiphosphate. Glucagon in various dilutions (10^{-8}–10^{-6} M final concentration) was added in 10-μl volumes, yielding a total reaction volume of 70 μl. The reaction was started by the addition of membranes resuspended in 0.25 M sucrose-1 mM EDTA-5 mM Tris-HCl, pH 7.5 (100 μg of membrane protein per tube), and tubes were incubated for 30 min at 30°C. The reaction was terminated by the addition of 100 μl of "stopping solution" containing 2.0 mM ATP, 0.5 mM cAMP, and 5,000–10,000 cpm of [^{3}H]cAMP as a recovery standard. Reaction tubes were then boiled for 3 min at 100°C, brought up to 1.0 ml with water, and applied sequentially to Dowex AG 50W-X4 (200–400 mesh, hydrogen form, Bio-Rad Laboratories) and alumina columns (32). Triplicates were usually within 5–15%, and recovery of [^{3}H]cAMP ranged between 70 and 95%.

CHEMICAL DETERMINATIONS: Protein was measured by the method of Lowry et al. (33) using BSA as a standard. Total sialic acids in plasma membrane subfractions were determined by the barbituric acid method (34). To avoid the interference of sucrose with the chemical reaction, we washed the membrane by repetitive resuspension in water and then repelleted them at 100,000 g_{ave} for 60 min. Membrane lipids were extracted according to Folch et al. (35) and total lipid phosphorus was estimated by the method of Bartlett (36). Individual phospholipid species were separated by two-dimensional thin-layer chromatography on Silica Gel 60 (Merck Chemical Div., Rahway, NJ) using the solvent systems described by Rouser et al. (37, 38). Standard phospholipids were run in parallel and the separated lipids were detected by iodine vapors. Individual spots were scraped and the lipid phosphorus were determined (36). Total and unesterified cholesterol were measured by gas-liquid chromatography with and without a hydrolysis step (39) using conditions as described previously (40).

SDS GEL ELECTROPHORESIS: SDS PAGE was carried out as described elsewhere (41) with the exception that a 1.5-mm thick slab gel apparatus with a linear 5–15% polyacrylamide gradient was used. Electrophoresis was carried out at room temperature at a constant current of 25 mA for ~13 h. Gels were fixed in 25% methanol/7% acetic acid and stained either with 2% Coomassie Blue in 50% methanol/14% acetic acid or alternatively by the silver stain technique as follows: the gel was soaked in 0.006% $NaMnO_4$ for 10 min, placed in 0.1% Na_2CO_3 for 10 min, and washed in H_2O for 5 min. The gel was incubated with 0.1% $AgNO_3$ for 10 min and rinsed with H_2O. Developer (30 g Na_2CO_3, 200 μl 37% formaldehyde per liter) was added and the gel was soaked until bands reached appropriate intensity. The development was stopped by washing the gel in 1% acetic acid.

Immunological Detection of SC

A specific antibody against the soluble biliary 80,000-mol-wt form of rat SC was raised in rabbits. Methods for the preparation, as well as characterization of the antibody, are described elsewhere (E. Sztul, manuscript in preparation). For detection of the various membrane forms of SC (42), a Western blot immuno-overlay technique was used. Upon completion of electrophoresis, gels were transferred to nitrocellulose (NC) filters (Schleicher and Schuell, Inc., Keene, NH) at room temperature at constant current (50 mA) for 4 h in a buffer containing 20% methanol. The filters were used immediately after transfer; they were quenched in 12.5 mg/ml hemoglobin (incubations for 2 h at room temperature under gentle agitation in the presence of 0.2% azide; reference 43) and then incubated with anti-SC sera (1:50 dilution in 12.5 mg/ml hemoglobin) overnight at room temperature. Filters were washed (2 × 10 min) with PBS, then with PBS containing 0.05% Nonidet P-40 (2 × 10 min), and rinsed with PBS (2 × 5 min). Adsorbed IgGs were detected by incubating the filter in ^{125}I-protein A (New England Nuclear) in 12.5 mg/ml hemoglobin (16 × 10^9 cpm ^{125}I/200 ml solution) for 4 h at room temperature followed by washing (as above), drying, and autoradiography.

Electron Microscopy

The LPM subfractions were resuspended in 0.25 M sucrose and fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. The samples were pelleted and postfixed in 1% osmium tetroxide. The specimens were then stained en bloc with 0.5% uranyl acetate in 0.15 M NaCl, dehydrated in graded ethanol solutions, and embedded in Spurr's medium. Ultrathin sections were prepared through several levels of the pellets with a diamond knife on an ultracut microtome (American Optical Corp., Southbridge, MA), stained with uranyl acetate and lead citrate, and examined with a Zeiss 10B electron microscope.

RESULTS

Presentation of the results is divided into the selective isolation of cLPM and blLPM from rat liver, the enzymatic, morphological, and chemical characterization of these two hepatocellular LPM subfractions, and the immunological detection of SC in blLPM and cLPM.

Isolation of LPM Subfractions

ISOLATION OF MIXED LPM: As demonstrated in Table I, ~30% of the homogenate's 5′-nucleotidase and Na^+K^+-ATPase activities was recovered in the initial crude nuclear pellet. In contrast, the recovery of alkaline phosphatase activity (a putative canalicular marker) amounted to 47%. During the subsequent zonal centrifugation of the "crude nuclear pellet", 70% of the loaded Na^+K^+-ATPase (Fig. 1*A*) and 52% of alkaline phosphatase activities (Fig. 1*B*) floated up to the 1.1972/1.1587 density interface whereas the majority of the mitochondrial cytochrome oxidase (76%) and the microsomal NADPH cytochrome *c* reductase (87%) were retained in the higher density sucrose cushions. The residual mitochondrial and microsomal membranes still contaminating the zonal LPM peak were largely removed during the two subsequent washing steps (see Materials and Methods). Since the repeated

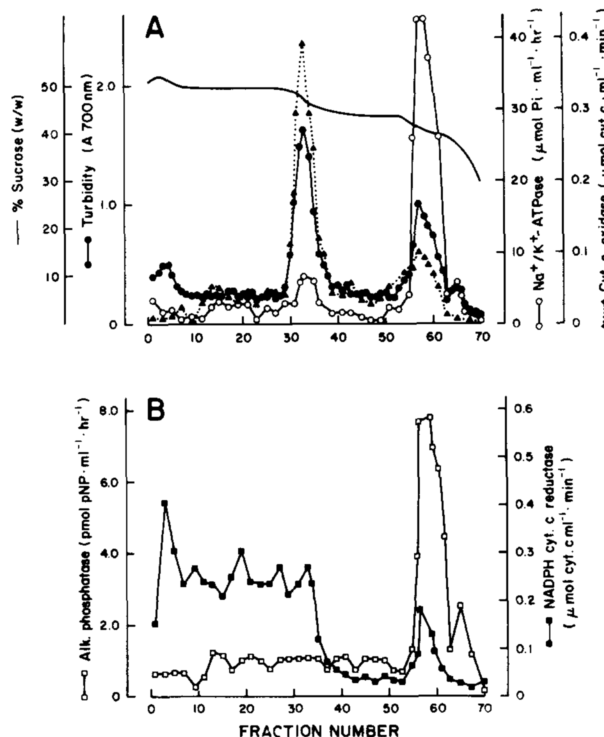

FIGURE 1 Rate zonal isolation of "mixed LPM". The initial crude nuclear pellet was resuspended in 56% sucrose and centrifuged in a discontinuous sucrose gradient system as described in Materials and Methods. After completion of the run, 15-ml fractions were collected and analyzed for mitochondrial (cytochrome c oxidase), microsomal (NADPH cytochrome c reductase; *B*), basolateral plasma membrane (Na^+K^+-ATPase; *A*), and canalicular plasma membrane (alkaline phosphatase; *B*) marker enzyme activities.

washing steps were required to achieve separation from intracellular organelles, the final recovery of mixed LPM was decreased to 12-17% (Table I). However, because of the large capacity of the TZ-28 zonal rotor, ~110 mg protein of mixed LPM could be isolated. We next subfractionated the mixed LPM using the selective basolateral localization of the $Na^{+}K^{+}$-ATPase (11, 12) as an initial marker to indicate separation of the canalicular and basolateral membrane domains.

SEPARATION OF cLPM AND blLPM: As illustrated in Fig. 2, after homogenization of the mixed LPM with the tight Dounce (50 up-and-down strokes), two enzymatically distinct LPM subfractions could be separated by isopycnic centrifugation in a linear sucrose gradient. Quantitatively, the majority of membrane fragments banded around a medium density of 1.19 as checked by turbidity measurements. These heavy membranes were enriched in $Na^{+}K^{+}$-ATPase and depleted in Mg^{++}-ATPase and alkaline phosphatase activities. In contrast, a considerable portion of the low density membranes (medium density 1.13, fractions 16-22) exhibited no $Na^{+}K^{+}$-ATPase activity but was highly enriched in canalicular marker enzyme activities. On the basis of these results we developed a discontinuous sucrose gradient system (see Materials and Methods) which permitted the reproducible isolation of a light density (<31% wt/wt sucrose), canalicular membrane subfraction usually completely devoid of any detectable $Na^{+}K^{+}$-ATPase activity (Table I). A heavy (34/38% wt/wt sucrose interface) subfraction of vesiculated basolateral membranes containing ~30% of the mixed LPMs $Na^{+}K^{+}$-ATPase activity could be simultaneously isolated. Quantitatively, the purified cLPM represented 11% and the vesiculated blLPM 19% of total mixed LPM protein. However, most of the total mixed LPM (48% of total protein) sedimented into the pellet as membrane sheets containing intact junctional complexes and desmosomes (Fig. 3). The 34% membranes represented a mixture of canalicular and basolateral LPM vesicles. This subfraction as well as the pellet were analyzed only to the extent required for a complete balance sheet of proteins and marker enzyme activities. As demonstrated in Table I, the total recoveries of protein and enzyme activities in the separated LPM subfractions were between 87 and 94% indicating no significant inactivation of the enzyme activities during the subfractionation procedure. Complete balance sheets were also performed for all additional enzyme data shown in Table II. Recoveries of enzyme activity ranged between 85 and 103% in all cases (data not shown).

Enzymatic Characterization of the Isolated LPM Subfractions

The specific activities for the various marker enzymes and their enrichment compared with homogenate (relative enrichment, RE) are summarized in Table II.

Most of the intracellular marker enzyme activities (Table II*A*) were depleted in the various LPM subfractions with respect to the homogenate with the exceptions of the lysosomal marker acid phosphatase in cLPM (RE = 1.1) and the inner mitochondrial membrane enzyme succinate cytochrome *c* reductase in blLPM (RE = 1.4). Since it has been estimated that lysosomes and mitochondria represent 2 and 16% of total rat liver homogenate protein whereas endoplasmic reticulum, Golgi, and peroxisomal membranes account for 24, 1, and 3%, respectively (44), it appears from the RE factors presented in Table II that microsomes and mitochondria form the major contaminants of the isolated LPM subfractions. For example, the RE value of 0.4 for NADPH cytochrome *c* reductase corresponds to a 9.6% (24 × 0.4) contamination of cLPM with microsomal membranes. Similarly, 22% (16 × 1.4) of total blLPM protein could be ac-

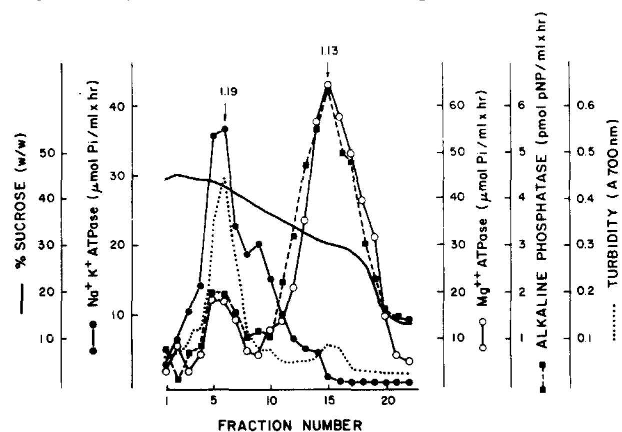

FIGURE 2 Isopycnic separation of canalicular and basolateral LPM. 1.5 ml of tightly homogenized mixed LPM (5.8 mg protein) was loaded on a linear sucrose gradient (28-47% wt/wt) and centrifuged at 40,000 rpm for 3 h (SW 41-rotor, Beckman Instruments Inc.). After the run, 22 fractions were collected from the bottom of the tubes and analyzed for turbidity and basolateral ($Na^{+}K^{+}$-ATPase) and canalicular (Mg^{++}-ATPase, alkaline phosphatase) plasma membrane marker enzyme activities. Numbers above arrows point to the sucrose densities of the enzyme activity peaks.

TABLE I
*Recovery of Protein and Plasma Membrane Marker Enzyme Activities**

	Protein (*n* = 12)	5'-Nucleotidase (*n* = 5)	$Na^{+}K^{+}$-ATPase (*n* = 12)	Alkaline phosphatase (*n* = 12)
	mg/g liver	*% distribution*	*% distribution*	*% distribution*
Homogenate	164.7 ± 12.5	100	100	100
Supernatant	—	63 ± 7	63 ± 20	45 ± 10
Crude nuclear pellet	—	32 ± 5	28 ± 12	47 ± 8
Mixed LPM	1.08 ± 0.22	12 ± 2	17 ± 3	15 ± 2
<31% (cLPM)	0.12 ± 0.03	5 ± 1	ND	5 ± 1
34%	0.18 ± 0.06	3 ± 1	3 ± 1	4 ± 1
34/38% (blLPM)	0.20 ± 0.06	1 ± 0.5	5 ± 1	2 ± 0.5
pellet	0.52 ± 0.10	2 ± 0.5	8 ± 2	2 ± 0.5

ND, not detectable.
* Data are given as mean ± SD with the numbers of experiments in parentheses.

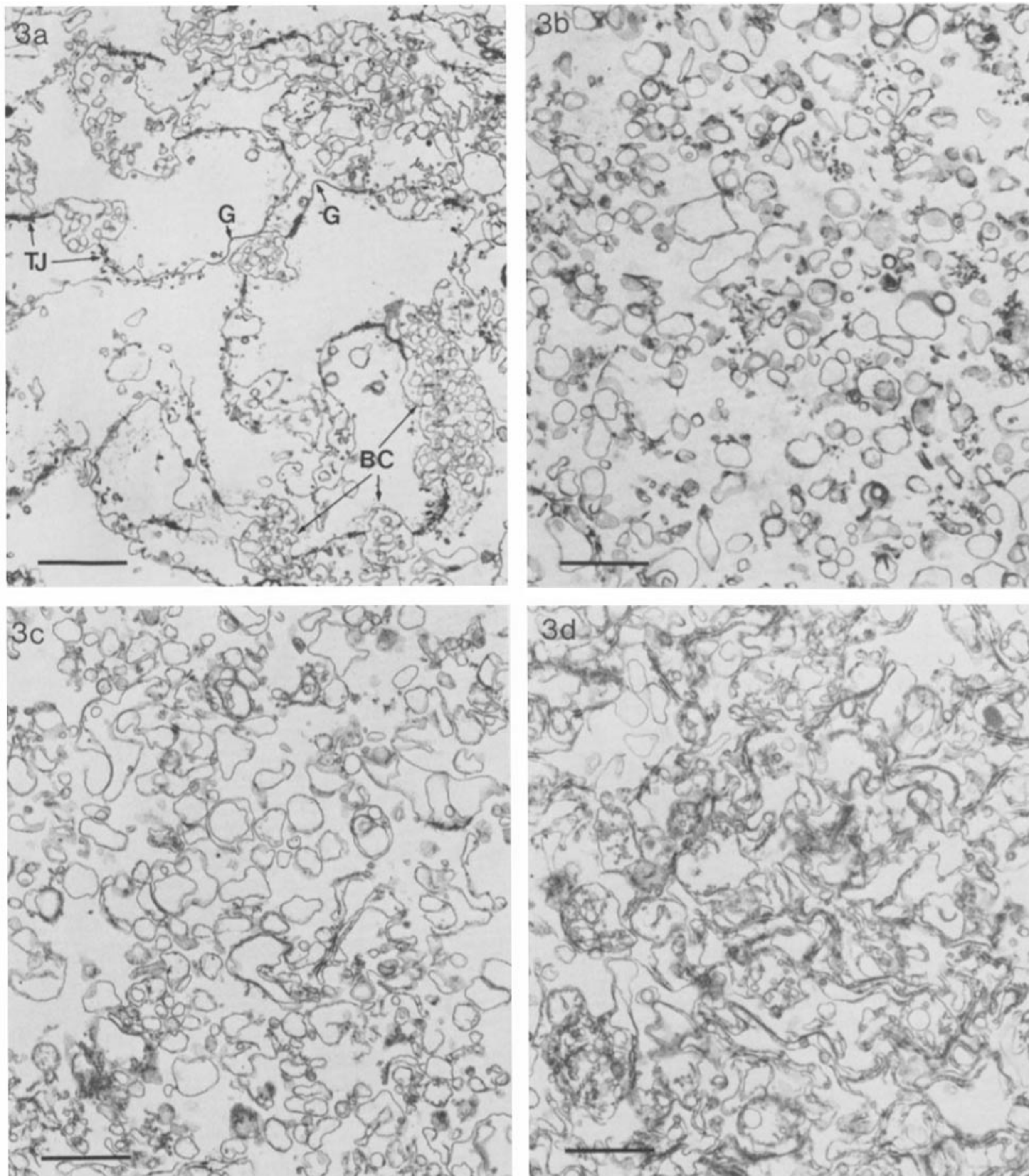

FIGURE 3 Electron micrographs of isolated rat LPM subfractions. (*a*) Mixed LPM with basolateral sheets, gap junctions (*G*), tight junctions (*TJ*), and intact bile canaliculi (*BC*). Bar, 2 μm. × 7,420. (*b*) Vesiculated cLPM subfraction. (*c*) blLPM vesicles containing some gap junctions and unbroken sheets. (*d*) Pellet consisting mainly of unbroken lateral membrane sheets and desmosomes. Bars (*b–d*), 1 μm. × 14,000.

counted for by inner mitochondrial membranes. In contrast, both Golgi and lysosomal contaminations of both LPM subfractions were consistently <3.0%. Various modifications of the sucrose gradients used for subfractionation did not further decrease the total contamination of cLPM (19%) and blLPM (26%) with intracellular organelles.

As further demonstrated in Table II*B*, the isolated cLPM contained no measurable Na^+K^+-ATPase activity whereas blLPM were 34-fold enriched in this enzyme activity. To test for substrate (ATP) inaccessibility, we also measured the Na^+K^+-ATPase activity after osmotic shock of cLPM in H_2O and in the presence of various concentrations of saponin (0.02–1.2 mg/mg protein) or digitonin (0.02–0.9 mg/mg protein). None of these treatments revealed any latent Na^+K^+-ATPase activity in cLPM (data not shown). Similar results were obtained if the protease inhibitor phenylmethylsulphonyl fluoride (1 mM) or diisopropylfluorophosphate (300 μM) was added to the various isolation media. Since Na^+K^+-

TABLE II
*Enzymatic Characterization of Isolated LPM Subfractions**

	Homogenate (Specific activity)[‡]	Mixed LPM (RE)[§]	cLPM (RE)	blLPM (RE)
A. Marker enzyme activities for intracellular organelles				
NADH cyt. c reductase (11)	7.91 ± 1.04	0.5 ± 0.2	0.7 ± 0.3	0.5 ± 0.2
Succinate cyt. c reductase (10)	0.75 ± 0.13	0.9 ± 0.3	0.1 ± 0.02	1.4 ± 0.3
Monoaminooxidase (8)	122.34 ± 44.62	0.5 ± 0.2	0.4 ± 0.2	0.3 ± 0.1
NADPH cyt. c reductase (8)	1.25 ± 0.32	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
Acid phosphatase (8)	0.30 ± 0.07	0.7 ± 0.3	1.1 ± 0.3	0.3 ± 0.2
Galactosyltransferase (8)	16.58 ± 3.72	0.1 ± 0.1	0.4 ± 0.4	0.2 ± 0.3
B. Plasma membrane marker enzyme activities				
Na^+K^+-ATPase (18)	0.68 ± 0.19	26 ± 10	ND	34 ± 5
Mg^{++}-ATPase (18)	1.62 ± 0.46	19 ± 5	83 ± 22	11 ± 3
Alkaline phosphatase (12)	0.39 ± 0.08	22 ± 9	71 ± 21	12 ± 4
Leucylnaphthylamidase (6)	3.63 ± 0.54	11 ± 2	48 ± 4	4 ± 1
γ-glutamyltranspeptidase (7)	0.07 ± 0.03	22 ± 9	60 ± 12	15 ± 5
Alkaline phosphodiesterase I (6)	4.50 ± 0.75	26 ± 5	116 ± 30	19 ± 7
5′-nucleotidase (5)	1.18 ± 0.07	17 ± 2	64 ± 5	11 ± 5

* Data are given as means ± SD with the number of experiments in parentheses.
‡ Specific activities are expressed as μmol product formed $\cdot mg^{-1} \cdot h^{-1}$ except for monoaminooxidase and galactosyltransferase for which the corresponding units are nmol kynuramine degraded $\cdot mg^{-1} \cdot h^{-1}$ and nmol galactose transferred $\cdot mg^{-1} \cdot h^{-1}$, respectively.
§ Relative enrichment is defined as the ratio of specific activity in the LPM to specific activity in the homogenate.

ATPase activity could be detected in as low as 0.3 μg blLPM protein whereas in cLPM no Na^+K^+-ATPase activity could be measured in up to 45 μg protein, it can be calculated that the maximal contamination of cLPM with basolateral membrane fragments was <0.7%. The purity of cLPM is further documented by the absence of any glucagon-stimulatable adenylate cyclase activity in this LPM subfraction (142.0 ± 12.7 without glucagon and 149.1 ± 35.5 pmol cAMP$\cdot mg^{-1} \cdot$ 30 min^{-1} in the presence of 10^{-8} M glucagon; mean ± SD; $n = 3$). In contrast, 10^{-8} M glucagon increased the adenylate cyclase activity from 161.1 ± 7.1 to 402.8 ± 58.1 pmol cAMP$\cdot mg^{-1} \cdot$ 30 min^{-1} (mean ± SD; $n = 3$) in blLPM. These findings corroborate several previous studies suggesting that the glucagon-stimulatable adenylate cyclase activity is exclusively confined to the basolateral domains of hepatocytes (10, 45). Besides Na^+K^+-ATPase and the glucagon-stimulatable adenylate cyclase activities, no other qualitative enzymatic differences between the two LPM subfractions were found. However, the activities of the putative canalicular marker Mg^{++}-ATPase (oligomycin insensitive), alkaline phosphatase, leucylnaphthylamidase, γ-glutamyltranspeptidase, alkaline phosphodiesterase I, and 5′-nucleotidase were considerably higher in cLPM (RE values of 48–116) as compared with blLPM (RE factors of 4–19). These findings clearly demonstrate an effective separation of basolateral membrane fragments from canalicular LPM.

Morphology of Isolated LPM Subfractions

The mixed LPM fraction showed the characteristic morphology of LPM prepared from low-spin crude nuclear pellets (10, 18, 46, 47). This fraction contained lateral membrane sheets with intact bile canaliculi, tight junctions, gap junctions, desmosomes, and free membrane vesicles (Fig. 3*a*). Attached fragments of the adjacent sinusoidal surface were also present. Intracellular organelles were rarely detected. After tight homogenization of this mixed LPM, a large proportion of the fraction was vesiculated; however, sheets of membranes attached to desmosomes and tight junctions were still present. Most of the membrane vesicles were retained in the three-step sucrose gradient during the subsequent high speed centrifugation whereas the bulk of membrane sheets sedimented to the bottom of the tubes. The cLPM subfraction mainly consisted of membrane vesicles of various sizes (Fig. 3*b*). Although the blLPM subfraction (Fig. 3*c*) was also predominantly vesiculated, some lateral membrane sheets with desmosomes and tight and gap junctions were regularly observed. The major part of the pellet (Fig. 3*d*) consisted of unbroken lateral membrane sheets containing desmosomes, tight junctions, and gap junctions. Numerous membrane vesicles were still present and occasionally unbroken bile canaliculi could be found in this fraction. The morphological data further confirm that a considerable portion of the hepatocellular lateral surface resists high mechanical shearing forces (10).

Biochemical Analysis of cLPM and blLPM

SIALIC ACIDS: The total membrane content of *N*-acetylneuraminic acids was twofold higher in cLPM than in blLPM (Table III*A*). These findings agree well with previous reports indicating that the concentration of sialic acids is higher in light than heavy LPM subfractions (10, 16).

LIPID ANALYSIS: In both LPM subfractions, >98% of the total cholesterol was unesterified. Compared with blLPM the cLPM were 2.2- and 1.8-fold enriched in cholesterol and total phospholipids, respectively, a difference consistent with their differential behavior in the sucrose density gradients. Both the molar ratios of cholesterol to phospholipids (0.54 for cLPM, 0.42 for blLPM) as well as the weight ratios (0.26 and 0.20 for cLPM and blLPM, respectively) were significantly different in the two LPM subfractions. Phospholipid analysis indicated that sphingomyelin was twofold enriched in cLPM as compared with blLPM (Table III*C*). Except for diphosphatidylglycerol, no other major differences in the phospholipid composition were found. Since diphosphatidylglycerol (cardiolipin) represents a mitochondria-specific phospholipid, its apparent higher concentration in blLPM compared with cLPM most probably reflects the higher mitochondrial contamination of blLPM (Table II). The lipid composi-

Table III
*Biochemical Analysis of cLPM and blLPM**

	cLPM	blLPM
A. Content of sialic acids (5)		
nmol/mg protein	86.2 ± 15.5	42.4 ± 5.2
B. Content of total cholesterol and phospholipids (5)		
Total cholesterol[‡]		
μmol/mg protein	0.45 ± 0.17	0.19 ± 0.08
mg/mg protein	0.17 ± 0.03	0.08 ± 0.02
Total phospholipids		
μmol/mg protein	0.83 ± 0.09	0.45 ± 0.04
mg/mg protein	0.65 ± 0.07	0.39 ± 0.03
C. Relative content of individual phospholipid species in % of total phospholipid phosphorus (5)		
Origin	trace[§]	trace
Phosphatidic acid	trace	trace
Diphosphatidylglycerol	0.6 ± 0.7	5.7 ± 1.1
Phosphatidylinositol	2.5 ± 1.0	2.1 ± 0.5
Phosphatidylserine	7.9 ± 1.3	7.4 ± 1.1
Lysophosphatidylcholine	0.7 ± 0.6	0.1 ± 0.2
Phosphatidylcholine	39.0 ± 2.4	38.7 ± 2.4
Sphingomyelin	23.9 ± 1.6	14.3 ± 2.1
Phosphatidylethanolamine	25.6 ± 1.6	31.8 ± 2.6

* All data are given as mean ± SD with the number of experiments in parentheses.
‡ Total cholesterol represents the sum of unesterified (>98%) and esterified cholesterol.
§ Detectable with iodine vapor but content of phosphorus not quantifiable.

tion of cLPM and blLPM as reported in this study agrees well with previous in vitro studies on light and heavy density LPM subfractions (48).

SDS GEL ELECTROPHORESIS ANALYSIS OF LPM SUBFRACTIONS: As demonstrated in Fig. 4, between 60 and 70 distinct protein bands could be resolved by SDS PAGE and detected after Coomassie Blue staining of the gel. At least three bands (130,000, 100,000, and 58,000 mol wt) demonstrate considerable enrichment in cLPM with the most prominent one laying in the molecular weight region of ~100,000. Moreover, several protein bands are selectively associated with blLPM and/or the pellet (~160,000, 80,000, 63,000, 62,000, 61,000, 60,000, 35,000, 28,000, and 27,000 mol wt): they most probably represent specific basolateral membrane proteins.

Differential Distribution of SC in cLPM and blLPM

As demonstrated in Fig. 5 (lanes *B* and *D*), the antibody preparation used was able to detect both the soluble 80,000-mol-wt form of SC in bile as well as the 116,000 and 94,000-mol-wt forms of the transmembrane SC in the mixed LPM fraction. When the latter was further subfractionated into cLPM and blLPM, the transmembrane form of SC was detected only in blLPM (Fig. 6). The lower limit of detection of SC in blLPM corresponded to 18.8 μg of protein. In contrast, no SC could be detected in as high as 187.5 μg of cLPM protein, which corresponded to the maximal loading capacity of the analytical method used. Similar results were obtained in the presence and absence of the protease inhibitor diisopropylfluorophosphate (300 μM) during the isolation of the LPM subfractions. These data indicate that intact SC is not an intrinsic component of bile canalicular membranes and further support the purity of the cLPM subfraction.

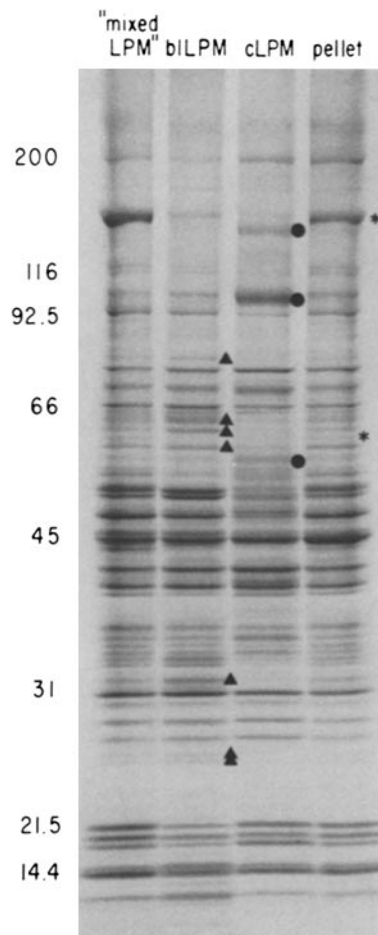

Figure 4 SDS PAGE of the various LPM subfractions. Samples (150 μg protein) were electrophoresed as described in Material and Methods and stained with Coomassie Blue. (●) Putative canalicular specific proteins (130,000, 100,000, and 58,000 mol wt). (▲) Protein bands concentrated in blLPM and virtually absent in cLPM (80,000, 63,000, 62,000, 60,000, 35,000, 28,000, and 27,000 mol wt). The asterisks designate protein bands concentrated in the pellet and virtually absent in cLPM (160,000 and 61,000 mol wt). (Molecular weight labels, × 10^{-3}.)

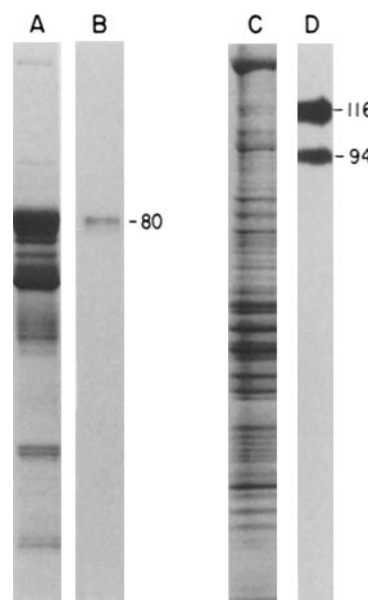

Figure 5 SC forms detected in bile and in the mixed LPM fraction. Bile was collected via a cannula inserted into the common bile ducts of rats. Mixed LPM were isolated as outlined in Materials and Methods. Protein samples of each (150 μg) were separated by SDS PAGE and either stained or transferred to NC filters and processed by immuno-overlay. (*A*) Coomassie Blue staining pattern of bile proteins. (*B*) Autoradiography of NC transfers of bile proteins after immuno-overlay. (*C*) Coomassie Blue staining pattern of mixed LPM. (*D*) Autoradiograph of NC transfers of mixed LPM proteins after immuno-overlay. (Molecular weight × 10^{-3}.)

DISCUSSION

A simple and reproducible method has been developed for the selective and simultaneous isolation of canalicular (cLPM) and basolateral (blLPM) plasma membrane vesicles from rat

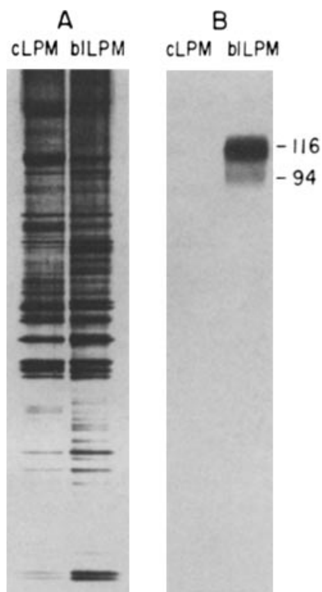

FIGURE 6 SC forms in cLPM and blLPM subfractions. LPM subfractions were isolated as in Material and Methods. Proteins of each (100 μg for both subfractions) were separated by SDS PAGE and either stained by the silver technique (*A*) or transferred to NC filters and processed by immunooverlay (*B*). (Molecular weight × 10^{-3}.)

liver. Both LPM subfractions are minimally contaminated with intracellular organelles. The cLPM subfraction is completely devoid of the basolateral marker Na^+K^+-ATPase and glucagon-stimulatable adenylate cyclase activity, but is 48- to 116-fold enriched in putative bile canalicular marker enzyme activities. Furthermore, cLPM contain twice as much total sialic acids, cholesterol, and sphingomyelin compared with blLPM. At least three canalicular- and nine basolateral-specific proteins have been detected after electrophoretic analysis of cLPM and blLPM. One of the integral plasma membrane proteins with known physiological function, the IgA-binding secretory component is exclusively localized in blLPM as demonstrated by immunochemical techniques. These data demonstrate that the described subcellular fractionation procedure represents a convenient method for routine high yield separation of canalicular from basolateral LPM vesicles in 1 d.

Several attempts have been made to separate canalicular and basolateral hepatocellular membrane domains from rat liver (10, 17–19, 45, 49–51). The most complete studies have been reported by Evans and co-workers, and indicate that intracellular organelle-free low and high density LPM subfractions are best separated initially from a "crude nuclear pellet" (1). However many of these isolation procedures are impractical for routine membrane work because of time consuming ultracentrifugation steps (e.g., up to 16 h in reference 17) and the use of unconventional zonal centrifugation procedures (16). Furthermore, all of the isolated light density subfractions still are significantly enriched in Na^+K^+-ATPase activity (18, 19, 45, 49–52). Na^+K^+-ATPase, however, is now thought to be exclusively localized at the basolateral surface of hepatocytes (11, 12). Therefore, the absence of any Na^+K^+-ATPase activity is an important prerequisite for claiming purity of a putative canalicular LPM subfraction. This goal was achieved in the present study after extensive modifications of a subfractionation procedure developed in this laboratory (18). The most important modifications introduced are the use of the TZ-28 zonal rotor to increase the total yield of mixed LPM 10-fold and a high speed separation of cLPM and blLPM vesicles in a new three-step discontinuous sucrose gradient system. In one isolation, between 9–16 mg cLPM protein free of any Na^+K^+-ATPase activity can be reproducibly prepared (18 out of 22 membrane isolations, Table II). The high recoveries during subfractionation (94%, Table I) as well as the persistent absence of measurable Na^+K^+-ATPase activity after osmotic shock of membranes and in the presence of saponin, digitonin, phenylmethylsulphonyl fluoride, and diisopropylfluorophosphate argue against inactivation and/or substrate inaccessibility of enzyme sites as the cause of undetectable Na^+K^+-ATPase in cLPM. Thus, in conjunction with the reported in vivo studies (11, 12), these data further confirm the absence of Na^+K^+-ATPase activity in rat canalicular LPM and exclude a significant contamination of cLPM with basolateral membrane components (calculated cross-contamination <0.7%). The latter conclusion is further supported by (*a*) the absence of glucagon-stimulatable adenylate cyclase activity in cLPM (see Results and reference 10), (*b*) the selective detection of several protein bands in blLPM and the pellet (Fig. 4), and (*c*) the absence of immunochemically detectable SC in cLPM (Fig. 6). In contrast, although the 34-fold enrichment in Na^+K^+-ATPase activity indicates that the majority of the blLPM vesicles derives from sinusoidal and lateral surface domains of the hepatocytes, the 4- to 19-fold enrichment of the canalicular markers in blLPM (Table II) suggests that this subfraction still is contaminated with canalicular LPM. Assuming an exclusive canalicular localization of the leucylnaphthylamidase (53), which is 12-fold higher enriched in cLPM than in blLPM (Table II), the calculated contamination of blLPM with cLPM is ~8%. It remains unclear at present why the various canalicular markers are unequally enriched in the isolated LPM subfractions. This may be partially explained by a differential subcellular distribution of some of the putative canalicular marker enzymes (54, 55). In addition, since alkaline phosphodiesterase I has been recently proposed as a marker for endosome-like vesicular structures (56), the possibility exists that cLPM still represent a more heterogenous subfraction than is indicated by the standard intracellular marker enzyme activities included in Table II. Clearly, the resolution of these questions has to await the more sensitive localization of the individual enzyme proteins in the isolated membrane vesicles by immunochemical techniques.

In agreement with some (10, 45, 48) but not all previous reports (18, 49, 51), we found higher contents of sialic acids, cholesterol, and sphingomyelin in cLPM than in blLPM (Table III). The high content of sialic acids corroborates the ultrastructural demonstration of an extensive "glycocalyx" on the surface of the bile canalicular membrane (7) and implies a strong negative surface charge within the bile canalicular lumen.

Several functional implications can be derived from the high cholesterol and high sphingomyelin contents of cLPM. Cholesterol has a condensing effect on the acyl chain region of lipid bilayers and thus membranes become more rigid and less permeable to water and small solutes such as glucose, K^+, and Ca^{++} (57, 58). Membrane cholesterol also inhibits Na^+K^+-ATPase and glucagon-stimulatable adenylate cyclase of erythrocytes and mixed LPM, respectively (59, 60), whereas sphingomyelin protects erythrocytic membranes against bile salt attack (61). Thus, the high cholesterol and sphingomyelin contents of cLPM most probably serve special roles in the regulation of bile secretory processes.

Both quantitative and qualitative differences are present in the protein patterns of cLPM and blLPM on Coomassie Blue stains of the SDS gels (Fig. 4). Since cLPM are practically free

of basolateral membrane components whereas blLPM presumptively are still contaminated with canalicular LPM, a marked concentration of protein bands in cLPM points to canalicular-specific plasma membrane proteins. On this basis the most prominent bile canalicular protein(s) was (were) found in the molecular weight region of ~100,000. Preliminary studies suggest that this (these) protein(s) selectively binds wheat germ agglutinin (data not shown). These characteristics are similar to a bile canalicular-specific antigen recently identified with monoclonal antibodies by others (62). Attempts are currently made to elucidate the functional role(s) of this major bile canalicular component.

Finally, the purified cLPM and blLPM subfractions were used to localize SC, a membrane receptor involved in transport of polymeric IgA (42, 63-65). This protein is synthesized as a transmembrane protein (66) in the rough endoplasmic reticulum, glycosylated in the Golgi complex, and then transported and inserted into the basolateral LPM (13). The SC exposed in the space of Disse binds plasma dimeric IgA and the complex is translocated across the cell to bile canaliculi via transcytosis (67). This transport is coupled to a proteolytic processing of the transmembrane form of SC into a soluble smaller form (still bound to dimeric IgA) which is then secreted into the bile canalicular lumen. It is currently unknown where the proteolytic cleavage of the transmembrane SC occurs in the cell, especially whether the transmembrane form of SC is intactly inserted into the bile canalicular membrane and then cleaved or whether the cleavage occurs prior to fusion of the transfer vesicles with the canalicular membrane. The fact that the transmembrane (116,000 and 94,000-mol-wt) form of SC could not be detected in cLPM (Fig. 6) indicates that intact SC is not an integral component of the bile canalicular membrane. Thus, the proteolytic processing of SC most probably occurs before the fusion of the transfer vesicles with the bile canalicular membranes although cleavage at cLPM followed by rapid secretion into bile cannot be definitely ruled out in the present study.

In summary, the data presented here confirm and extend the evidence for a considerable functional and structural polarity of the various hepatocellular surface domains. To our knowledge this is the first study in which complete Na^+K^+-ATPase activity and SC-free cLPM vesicles have been reproducibly separated from blLPM of rat liver. Thus, the presented procedure results in the most highly purified cLPM subfraction so far available. The isolation method is carried out with commercially available equipment and results in sufficient yields of membranes to permit extensive functional studies (68, 69). Therefore, the described technique should facilitate further investigations of the functional polarity of the hepatocellular surface domains especially in regard to one of the most important physiological functions of the liver, the formation and canalicular secretion of bile acids, organic anions, and other biliary components.

We thank Dr. A. Stewart for the determination of the adenylate cyclase activity; L. Barrett, A. Meier-Abt, and R. Allen for their excellent technical assistance; Oi Cheng Ng for performing the electron microscopy; and D. Smith for the efficient typing of the manuscript. We are most grateful to Dr. George Palade for critically reading the manuscript.

This work was supported by U.S. Public Health Service grants GM-27303 to E. Sztul and AM-25636, AM-31493, and AM-17433. Dr. P. J. Meier is supported by a postdoctoral fellowship of the Swiss National Science Foundation (Nr. 83.907.0.81) and the Swiss Foundation of Experimental Medicine and Biology. Dr. A. Reuben is a recipient of U.S. Public Health Service Clinical Investigator Award 1 K08 AM0 1111-01.

Received for publication 11 July 1983, and in revised form 28 October 1983.

REFERENCES

1. Evans, W. H. 1980. A biochemical dissection of the functional polarity of the plasma membrane of the hepatocyte. *Biochim. Biophys. Acta.* 604:27–64.
2. Boyer, J. L. 1980. New concepts of mechanisms of hepatocyte bile formation. *Physiol. Rev.* 60:303–326.
3. Wall, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. *Cell.* 21:79–93.
4. Renston, R. H., D. G. Maloney, A. L. Jones, G. T. Hradek, K. Y. Wong, and J. D. Goldfine. 1980. Bile secretory apparatus: evidence for a vesicular transport mechanism for proteins in the rat, using horseradish peroxidase and [^{125}I]insulin. *Gastroenterology.* 78:1373–1388.
5. Goodenough, D. A., and J. P. Revel. 1970. A fine structural analysis of intercellular junctions in the mouse liver. *J. Cell Biol.* 45:272–290.
6. Blitzer, B. L., and J. L. Boyer. 1982. Cellular mechanisms of bile formation. *Gastroenterology.* 82:346–357.
7. Oda, M., V. M. Price, M. M. Fisher, and M. J. Phillips. 1974. Ultrastructure of bile canaliculi, with special reference to the surface coat and the pericanalicular web. *Lab Invest.* 31:314–323.
8. Hubbard, A. L., D. A. Wall, and A. Ma. 1983. Isolation of hepatocyte plasma membranes. I. Presence of the three major domains. *J. Cell Biol.* 96:217–229.
9. Jones, A. L., R. H. Renston, and S. J. Burwen. 1982. Uptake and intracellular disposition of plasma derived proteins and apoproteins by hepatocytes. *In* Progress in Liver Diseases. H. Popper and T. Schaffner, editors. Grune and Stratton, Inc., New York. 7:51–69.
10. Wisher, M. H., and W. H. Evans. 1975. Functional polarity of the rat hepatocyte surface membrane. Isolation and characterization of plasma-membrane subfractions from the blood-sinusoidal, bile-canalicular and contiguous surfaces of the hepatocyte. *Biochem. J.* 146:375–388.
11. Blitzer, B. L., and J. L. Boyer. 1978. Cytochemical localization of Na^+K^+-ATPase in the rat hepatocyte. *J. Clin. Invest.* 62:1101–1104.
12. Latham, P. S., and M. Kashgarian. 1979. The ultrstructural localization of transport ATPase in the rat liver at non-bile canalicular plasma membrane. *Gastroenterology.* 76:988–996.
13. Mullock, B. M., R. H. Hinton, M. Dobrota, J. Peppard, and E. Orlans. 1980. Distribution of secretory component in hepatocytes and its mode of transfer into bile. *Biochem. J.* 190:819–826.
14. Renston, R. H., A. L. Jones, W. D. Chirstiansen, G. T. Hradek, and B. J. Underdown. 1980. Evidence for a vesicular transport mechanism in hepatocytes for biliary secretion of immunoglobulin A. *Science (Wash. DC).* 208:1276–1278.
15. Takahashi, I., P. K. Nakane, and W. R. Brown. 1982. Ultrastructural events in the translocation of polymeric IgA by rat hepatocytes. *J. Immunol.* 128:1181–1187.
16. Evans, W. H. 1970. Fractionation of liver plasma membranes prepared by zonal centrifugation. *Biochem. J.* 166:833–842.
17. Touster, O., N. N. Aronson, J. T. Dulaney, and H. Hendrickson. 1970. Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* 47:604–618.
18. Boyer, J. L., R. M. Allen, and Oi Cheng Ng. 1983. Biochemical separation of Na^+K^+-ATPase from a "purified" light density, "canalicular"-enriched plasma membrane fraction from rat liver. *Hepatology (Baltimore).* 3:18–28.
19. Inoue, M., R. Kinne, T. Tran, L. Biempica, and J. M. Arias. 1983. Rat liver canalicular membrane vesicles. Isolation and topological characterization. *J. Biol. Chem.* 258:5183–5188.
20. Meier, P. J., M. C. Duffy, and J. L. Boyer. 1982. Isolation of purified canalicular plasma membranes in high yield from rat liver. *Hepatology (Baltimore).* 2:737*a*. (Abstr.)
21. Avruch, J., and D. F. H. Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated fat cells. *Biochim. Biophys. Acta.* 233:334–347.
22. Scharschmidt, B. F., E. B. Keeffe, N. M. Blankenship, and R. K. Ockner. 1979. Validation of a recording spectrophotometric method for measurement of membrane associated Mg^{++} and Na^+K^+-ATPase activity. *J. Lab. Clin. Med.* 93:790–799.
23. Keeffe, E. B., B. F. Scharschmidt, N. M. Blankenship, and R. K. Ockner. 1979. Studies of relationships among bile flow, liver plasma membrane Na^+K^+-ATPase, and membrane microviscosity in the rat. *J. Clin. Invest.* 64:1590–1598.
24. Goldbarg, J. A., and A. M. Rutenburg. 1958. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. *Cancer (Phila.).* II:283–291.
25. Orlowski, M., and A. Meister. 1963. γ-Glutamyl-*p*-nitroanilide: a new convenient substrate for determination and study of L- and D-γ-glutamyltranspeptidase activities. *Biochim. Biophys. Acta.* 73:676–679.
26. Razzell, W. E. 1961. Tissue and intracellular distribution of two phosphodiesterases. *J. Biol. Chem.* 236:3028–3030.
27. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. *J. Cell Biol.* 32:415–438.
28. Weissbach, H., T. E. Smith, J. W. Daly, B. Witkop, and S. Udenfriend. 1960. A rapid spectrophotometric assay of monoaminooxidase based on the rate of disappearance of kynuramine. *J. Biol. Chem.* 235:1160–1163.
29. Rothstein, T. L., and J. J. Blum. 1973. Lysosomal physiology in tetrahymena. I. Effect of glucose, acetate, pyruvate and carmine on intracellular content and extracellular release of three acid hydrolases. *J. Cell Biol.* 57:630–641.
30. Fleischer, B., and M. Smigel. 1978. Solubilization and properties of galactosyltransferase and sulfotransferase activities of Golgi membranes in Triton X-100. *J. Biol. Chem.* 253:1632–1638.
31. Stewart, A. F., K. L. Insogna, D. Goltzman, and A. E. Broadus. 1983. Identification of adenylate cyclase stimulating activity and cytochemical glucose-6-phosphate dehydrogenase stimulating activity in extracts of tumors from patients with humoral hypercal-

cemia of malignancy. *Proc. Natl. Acad. Sci. USA.* 80:1454–1458.
32. Solomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* 58:541–548.
33. Lowry, O. H., H. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
34. Aminoff, D. 1961. Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* 81:384–392.
35. Folch, J., M. Lees, and G. H. S. Stanley. 1956. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497–509.
36. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–471.
37. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* 5:494–496.
38. Bauman, A. J., R. E. Cameron, G. Kritchevsky, and G. Rouser. 1966. Detection of phthalate esters as contaminants of lipid extracts from soil samples stored in standard soil bags. *Lipids.* 1:85-86.
39. Ishikawa, T. T., J. MacGee, J. A. Morrison, and C. J. Glueck. 1974. Quantitative analysis of cholesterol in 5 to 20 μl of plasma. *J. Lipid Res.* 15:286–291.
40. Reuben, A., K. E. Howell, and J. L. Boyer. 1982. Effects of taurocholate on the size of mixed lipid micelles and their associations with pigment and proteins in rat bile. *J. Lipid Res.* 23:1039–1052.
41. Howell, K. E., and G. E. Palade. 1982. Hepatic Golgi fractions resolved into membrane and content subfractions. *J. Cell Biol.* 92:822–832.
42. Kuehn, L. C., and J. P. Kraehenbuhl. 1979. Role of secretory component, a secreted glycoprotein, in the specific uptake of IgA dimer by epithelial cells. *J. Biol. Chem.* 254:11072–11081.
43. Gershoni, J. M., and G. E. Palade. 1982. Electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to a positively charged membrane filter. *Anal. Biochem.* 124:396–405.
44. Neville, D. M. 1976. The preparation of cell surface membrane enriched fractions. *In* Biochemical Analysis of Membranes. A. H. Maddy, editor. Chapman and Hall, London. 27–54.
45. Toda, G., H. Oka, O. Toshitsugu, and Y. Ikeda. 1975. Subfractionation of rat liver plasma membrane. Uneven distribution of plasma membrane-bound enzymes on the liver cell surface. *Biochim. Biophys. Acta.* 413:52–64.
46. Neville, D. M. 1960. The isolation of a cell membrane fraction from rat liver. *J. Biophys. Biochem. Cytol.* 8:413–422.
47. Emmelot, P., and C. J. Bos. 1966. Studies on plasma membranes. III. Mg^{++}-ATPase, (Na^+,K^+-Mg^{++})-ATPase and 5'-nucleotidase activity of plasma membranes isolated from rat liver. *Biochim. Biophys. Acta.* 120:369–382.
48. Kremmer, T., M. H. Wisher, and W. H. Evans. 1976. The lipid composition of plasma membrane subfractions originating from the three major functional domains of the rat hepatocyte cell surface. *Biochim. Biophys. Acta.* 455:655–664.
49. Fisher, M. M., D. L. Bloxam, M. Oda, M. J. Phillips, and I. M. Yousef. 1975. Characterization of rat liver cell plasma membranes. *Proc. Soc. Exp. Biol. Med.* 150:177–184.
50. Kramer, W., U. Bickel, H. P. Buscher, W. Gerok, and G. Kurz. 1982. Bile salt binding polypeptides in plasma membranes of hepatocytes revealed by photoaffinity labelling. *Eur. J. Biochem.* 129:13–24.
51. Scharschmidt, B. F., and E. B. Keeffe. 1981. Isolation of a rat liver plasma membrane fraction of probable canalicular origin. Preparative technique, enzymatic profile, composition, and solute transport. *Biochim. Biophys. Acta.* 646:369–381.
52. Poupon, R. E., and W. H. Evans. 1979. Biochemical evidence that Na^+K^+-ATPase is located at the lateral region of the hepatocyte surface membrane. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 38:134–143.
53. Roman, L. M., and A. L. Hubbard. 1983. A domain-specific marker for the hepatocyte plasma membrane: localization of leucine aminopeptidase to the bile canalicular domain. *J. Cell Biol.* 96:1548–1558.
54. Stanley, K. K., M. R. Edwards, and J. P. Luzio. 1980. Subcellular distribution and movement of 5'-nucleotidase in rat cells. *Biochem. J.* 186:59–69.
55. Farquhar, M. G., J. J. M. Bergeron, and G. E. Palade. 1974. Cytochemistry of Golgi fractions prepared from rat liver. *J. Cell Biol.* 60:8–25.
56. Smith, G. D., and T. J. Peters. 1982. The localization in rat liver of alkaline phosphodiesterase to a discrete organelle implicated in ligand internalization. *Biochim. Biophys. Acta.* 716:24–30.
57. Blok, M. C., L. L. M. Van Deenen, and J. De Gier. 1977. The effect of cholesterol incorporation on the temperature dependence of water permeation through liposomal membranes prepared from phosphatidylcholine. *Biochim. Biophys. Acta.* 464:509–518.
58. Storch, J., D. Schachter, M. Inoue, and A. W. Wolkoff. 1983. Lipid fluidity of hepatocyte plasma membrane subfractions and their differential regulation by calcium. *Biochim. Biophys. Acta.* 727:209–212.
59. Yeagle, P. L. 1983. Cholesterol modulation of Na^+ K^+-ATPase ATP hydrolyzing activity in human erythrocyte. *Biochim. Biophys. Acta.* 727:39–44.
60. Whetton, A. D., L. M. Gordon, and M. D. Houslay. 1983. Elevated membrane cholesterol concentrations inhibit glucagon-stimulated adenyl cyclase. *Biochem. J.* 210:437–449.
61. Lowe, P. J., and R. Coleman. 1981. Membrane fluidity and bile salt damage. *Biochim. Biophys. Acta.* 640:55–65.
62. Hubbard, A. L., and L. B. Braiterman. 1982. Monoclonal antibodies to the major hepatocyte cell surface domains. *J. Cell Biol.* 95 (2, Pt. 2):97*a*. (Abstr.)
63. Socken, D. J., K. N. Jeejeebhoy, H. Barzin, and B. J. Underdown. 1979. Identification of secretory component as an IgA receptor on rat hepatocytes. *J. Exp. Med.* 50:1538–1548.
64. Delacroix, D. L., H. J. F. Hodgson, A. McPherson, C. Dive, and J. P. Vaerman. 1982. Selective transport of polymeric immunoglobulin A in bile. Quantitative relationships of monomeric and polymeric immunoglobulin A, immunoglobulin M, and other proteins in serum, bile, and saliva. *J. Clin. Invest.* 70:230–241.
65. Orlans, E., J. Peppard, J. F. Fry, R. H. Hinton, and B. M. Mullock. 1979. Secretory component as the receptor for polymeric IgA on rat hepatocytes. *J. Exp. Med.* 150:1577–1581.
66. Mostov, K. E., J. P. Kraehenbuhl, and G. Blobel. 1980. Receptor-mediated transcellular transport of immunoglobulin: synthesis of secretory component as multiple and larger transmembrane forms. *Proc. Natl. Acad. Sci. USA.* 77:7257–7261.
67. Mullock, B. M., R. H. Hinton, M. Dobrota, J. Peppard, and E. Orlans. 1979. Endocytic vesicles in liver carry polymeric IgA from serum to bile. *Biochim. Biophys. Acta.* 587:381–391.
68. Gordon, E. R., P. J. Meier, C. Goresky, and J. L. Boyer. 1983. Characteristics and subcellular site of bilirubin diglucuronide formation in rat liver. *Gastroenterology.* 84:1373*a*. (Abstr.)
69. Meier, P. J., and J. L. Boyer. 1983. The electrical membrane potential is a driving force for taurocholate (TC) excretion into bile canaliculi. *Hepatology (Baltimore).* 3:860*a*. (Abstr.)