

Isolation of Rat Hepatocyte Plasma Membranes.

I. Presence of the Three Major Domains

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ABSTRACT A rat liver plasma membrane preparation was isolated and characterized both biochemically and morphologically. The isolation procedure was rapid, simple and effective in producing a membrane fraction with the following biochemical characteristics: ~40-fold enrichment in three plasma membrane markers, 5'-nucleotidase, alkaline phosphodiesterase I (both putative bile canalicular membrane enzymes), and the asialo-glycoprotein (ASGP) receptor (a membrane glycoprotein present along the sinusoidal front of hepatocytes); a yield of each of these plasma membrane markers that averaged ~16%; and minimal contamination by lysosomes, nuclei, and mitochondria, but persistent contamination by elements of the endoplasmic reticulum. Morphological analysis of the preparation revealed that all three major domains of the hepatocyte plasma membrane (sinusoidal, lateral, and bile canalicular) were present in substantial amounts. The identification of sinusoidal membrane was further confirmed when ASGP binding sites were localized predominantly to this membrane in the isolated PM using electron microscope autoradiography. By morphometry, the sinusoidal front membrane accounted for 47% of the total membrane in the preparation, whereas the lateral surface and bile canalicular membrane accounted for 6.8% and 23% respectively. This is the first report of such a large fraction of sinusoidal membrane in a liver plasma membrane preparation.

The liver parenchymal cell (the hepatocyte) is a polarized epithelial cell whose surface is composed of three functionally and morphologically distinct domains. The sinusoidal front (SF)¹, specialized for exchange of metabolites with the blood, is characterized by irregular microvilli extending into the space of Disse and by numerous coated pits (1). The lateral surface (LS), which is contiguous to a neighboring hepatocyte LS, is specialized (at least in part) for cell attachments and cell-cell communication, and thus is marked by junctional elements such as tight junctions, desmosomes, and gap junctions (e.g. reference 2). The bile canalicular (BC) front, which is separated from the LS by tight junctions, is specialized for bile secretion and is characterized by numerous microvilli (e.g. reference 3).

These functional and morphological differences among the domains of the hepatocyte cell surface must be matched by compositional differences of membrane components. However, no satisfactory biochemical comparison of the domains has been performed to date.

Using a stereological approach, Weibel and colleagues (4-6) have reported that the SF of hepatocytes constitutes ~72% of the total cell's surface area (which is 5,200 μm^2), the LS 15%, and the BC front 13%. Rat liver plasma membrane preparations isolated by others (e.g. references 7-12) have been enriched primarily in the last two domains (LS and BC), which together comprise only 30% of the total surface area of the hepatocyte. Thus, it is necessary to develop isolation procedures that yield a final plasma membrane fraction that includes substantial portions of all three domains, including the SF. Successful isolation of SF membrane, either in continuity with the others or as a separate fraction, has not been achieved, largely because the microvilli of this membrane yield a population of smooth-surfaced vesicles when the tissue is homogenized. Such vesicular structures cannot be physically separated or morphologically distinguished from "smooth microsomes" derived from

¹ The abbreviations used in this article are as follows: sinusoidal front (SF), lateral surface (LS), bile canalicular front (BC), 5' adenosine monophosphate (5' AMP), sucrose/Tris-HCl/MgCl₂ (STM), plasma membrane (PM), trichloroacetic acid (TCA), alkaline phosphodiesterase I (APDE I), asialo-glycoprotein (ASGP), endoplasmic reticulum (ER), 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), asialo-orosomucoid (ASOR), orosomucoid (OR).

intracellular membrane or the plasma membrane fragments of nonparenchymal cells. Although isolation of putative SF subfractions has been reported, the evidence is based only on quantitative differences in marker enzyme activities whose localizations to particular domains have not been established (13, 14).

Our goal has been to isolate from liver a plasma membrane fraction that is representative of the entire hepatocyte cell surface for use in subsequent subfractionation studies. In the first paper we present a method that yields rat liver plasma membrane sheets containing each of the three domains in substantial amount. We have characterized this preparation both morphologically and biochemically. An important part of our initial characterization has been to identify molecular constituents of the preparation. In the accompanying paper (15) we report the identification of three components of the liver cytoskeleton that are associated with the isolated plasma membrane. Preliminary reports of this work have been presented (16, 17).

MATERIALS AND METHODS

Materials

Sucrose (ultra-pure) was obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY); p-nitrophenyl thymidine mono-phosphate, cytochrome c, glucose-6-phosphate (Na salt), alkaline phosphatase substrate, p-nitrophenyl- β -N-acetyl glucosaminide, agarose, and Tris base from Sigma Chemical Co. (St. Louis, MO); protein reagent from Bio-Rad Laboratories (Richmond, CA); 5'-adenosine monophosphate (5'AMP), NADPH, NADH, and ferritin (A grade) from Calbiochem-Behring Corp. (American Hoechst Corp., San Diego, CA); N,N-dimethyl-1, 3-propane diamine from Eastman Organic Chemicals Div., Eastman Kodak Co. (Rochester, NY); glutaraldehyde and osmium tetroxide (OsO_4) from Electron Microscopy Sciences (Fort Washington, PA); EPON 812 and Poly Bed 812 from Polysciences, Inc. (Warrington, PA); and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC) from Ott Chemical Co. (Muskegon, MI). All other chemicals were reagent grade. Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA).

Isolation of Plasma Membranes

All sucrose solutions were prepared 24–48 h before use and their densities determined at room temperature with an Abbé refractometer. The solutions were filtered (0.22 μm for 0.25 M and 1.2 μm for 1.42 or 2.0 M solutions) and stored at 4°C after again measuring the density and pH of each.

Male Sprague-Dawley rats (120–170 g) were starved for 16–24 h and killed by decapitation. Livers were excised intact and perfused through the portal vein with ice-cold 0.154 M NaCl until they blanched. All subsequent procedures were carried out at 0–4°C. Individual livers were weighed, minced with scissors, added to 4–4.5 volumes of 0.25 M STM (0.25 M sucrose/5 mM Tris HCl, pH 7.2–7.6/0.5 or 1.0 mM MgCl_2), and homogenized in 10 up-and-down strokes in a 40-ml Dounce-type glass homogenizer with a loose-fitting pestle. Five to seven livers were routinely used and weighed a total of 30–40 g. The homogenate was adjusted to 20% (liver wet weight to total volume) with 0.25 M STM and filtered through four layers of moistened gauze. The filtrate was centrifuged (25–30 ml per 50-ml plastic tube) at 280 g for 5 min (Step 1, 1,100 rpm in IEC PR-6000 or Beckman TJ-6R). The supernate was saved and the pellet was resuspended by three strokes of the loose Dounce in 0.25 M STM to one-half the initial homogenate volume. The suspension was again centrifuged as above. The first and second supernates were combined and centrifuged (25–30 ml per 50-ml tube) at 1,500 g for 10 min (Step 2, 2,600 rpm). The resulting pellets were pooled and resuspended by three strokes of the loose Dounce in ~1–2 ml of 0.25 M STM per gram of liver (initial wet weight). 2.0 M STM was added to obtain a density of 1.18 g/cm^3 (1.42 M, $n = 1.4010$) and sufficient 1.18 g/cm^3 STM was added to bring the volume to approximately twice that of the original homogenate (i.e., 10% wt/vol). 35-ml aliquots of the sample were added to cellulose nitrate tubes and overlaid with 2–4 ml 0.25 M sucrose. After centrifugation for 60 min at 82,000 g_{av} in a Beckman L5-65 centrifuge (Step 3, 25,000 rpm, SW 28 rotor, no brake), the pellicle at the interface was collected with a blunt-tipped Pasteur pipette and resuspended in a loose Dounce in sufficient 0.25 M sucrose to obtain a density of $\approx 1.05 \text{ g}/\text{cm}^3$. This suspension was centrifuged at 1,500 g for 10 min

(Step 4) and the final pellet (designated the plasma membrane [PM] fraction) resuspended in 0.25 M sucrose in a loose Dounce. Measured aliquots of each fraction throughout the procedure, starting with the filtered homogenate, were saved for subsequent enzymatic and chemical analyses.

Assays

All assays except cytochrome oxidase were performed on fractions stored at -70°C for up to one month. On several occasions measurements of fresh and frozen-thawed samples were compared and no significant differences were observed. Cytochrome oxidase was assayed on samples held at 4°C for no more than 8 h.

ENZYME ASSAYS: 5'-nucleotidase activity was measured in a final volume of 0.5 ml at 37°C in 90 mM Tris-HCl, pH 8.0, to which was added 5 mM 5' AMP and 10 mM MgCl_2 (18). Released phosphate was measured according to Ames (19) against a K_2HPO_4 standard. When detergents or other substances that interfered with the phosphate determination were present in samples, a radioactive 5'-nucleotidase assay was used (20). Alkaline phosphodiesterase I (APDE I) was assayed at 37°C in 0.04 M Na carbonate buffer, pH 10.5, 0.1% Triton X-100 with 2 mM thymidine-5'-monophospho-p-nitrophenyl ester as substrate. The reaction was stopped with cold 10% TCA and the released p-nitrophenol measured against a standard at 410 nm after addition of an alkaline reagent containing 133 mM glycine, 83 mM Na_2CO_3 and 67 mM NaCl, pH 10.7 (21). Alkaline phosphatase was measured according to Ray (22), except that the reaction was stopped with alkaline reagent, and the released p-nitrophenol was measured as described for APDE I. β -N-acetyl-glucosaminidase was measured at 37°C in 100 mM Na acetate, pH 5.0, 0.1% Triton X-100 using 8 mM p-nitrophenyl- β -N-acetylglucosaminide. The reaction was stopped by addition of 3 ml of alkaline reagent, and released p-nitrophenol was measured at 410 nm. Cytochrome oxidase was assayed according to Cooperstein and Lazarow (23) as modified by Peters et al. (24). NADH (or NADPH)-cytochrome c reductase was assayed by the method of Kreibich et al. (25). Asialoglycoprotein (ASGP) binding activity was assayed in the absence of detergent so that only the accessible² ASGP receptor was measured. Samples were incubated at 4°C or 23°C for 15–60 min in a volume of 0.4 ml that contained 12.5 mM Na HEPES, pH 7.4, 6.25 mM CaCl_2 , 190 mM NaCl, 0.6% BSA, and ¹²⁵I-asialo-orosomucoid (ASOR, $\sim 0.1 \mu\text{g}$ at $5\text{--}18 \times 10^6 \text{ cpm}/\mu\text{g}$, prepared and iodinated as described in reference 29). Binding was terminated by addition of 5 ml of a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM CaCl_2 , 300 mM NaCl and 0.1% BSA. The suspension was immediately filtered under gentle vacuum onto glass fiber disks (Whatman GF/C; Whatman, Inc., Clifton, NJ), the assay tubes were rinsed twice with 5 ml of cold rinse buffer, the filters rinsed three times with a total volume of 30 ml rinse buffer, and the radioactivity retained on the filters was measured (Beckman Biogamma or Model 4000; Beckman Instruments, Inc., Fullerton, CA). Nonspecific binding was determined by the addition of 100-fold excess unlabeled ASOR to the above incubation mix and was generally $\leq 10\%$ of the total binding observed. Galactosyl and sialyl transferases were assayed according to Howell et al. (30) and Bretz et al. (31) respectively.

CHEMICAL DETERMINATIONS: Protein was determined according to Lowry (32) using bovine serum albumin as a standard or according to Bradford (33) using bovine gamma globulin as a standard. RNA and DNA determinations were performed according to published methods (34, 35).

Electron Microscopy

Equal volumes of a sample (50–100 μg protein) and 3% glutaraldehyde in 0.1 M Na cacodylate, pH 7.4, were mixed, incubated for 30 min on ice, and the suspension was centrifuged in 0.8-ml cellulose nitrate tubes (SW 50.1 rotor, Beckman Instruments, Inc.). The resulting pellets were postfixed in OsO_4 and stained *en bloc* with uranyl acetate as previously described for liver tissue (36). The pellets were dehydrated through a graded series of alcohols and released from the tube by immersion in propylene oxide, which dissolved the cellulose nitrate. The pellets were embedded in Epon or Poly Bed 812 in an oriented

² Our own recent studies (D. A. Wall and A. L. Hubbard, submitted) as well as earlier reports (26) have indicated that substantial amounts of ASGP receptor activity in liver homogenates and subcellular fractions reside in "latent" sites that are revealed only by the addition of detergents. However, there is measurable binding activity in homogenates in the absence of detergent (26), which is due primarily to PM receptors. The presence of ASGP receptors in the SF domain of hepatocytes has been demonstrated by their function in the endocytosis of ASGPs from the circulation (references 1, 27) and by use of specific EM tracers (28).

fashion such that the entire thickness of the pellet was present in each section. Thin sections were stained with uranyl acetate then lead citrate and examined in either a Philips 301 or a Zeiss 10A electron microscope at 80 kV.

Morphometry

Six separate PM preparations were used for morphometric analysis. In five preparations, only PM present in the top of the pellets was scored, because only in this region were the individual sheets sufficiently separated to permit clear domain identification. In the sixth preparation, PM was embedded in agarose according to the method of deCamilli et al. (submitted for publication) which produced adequate separation of sheets and thus allowed analysis of a representative sample of all PM. Random pictures were taken at $\times 9,100$ and magnified to $\times 24,600$. A grid composed of a series of lines ($d = 1 \text{ cm}$ at $\times 24,600$) was laid over each micrograph and the intersections between a line and a membrane (identified as to domain as shown in Fig. 2) were tabulated. The total number of intersections is directly proportional to the surface area of membrane in the preparation (37). >850 intersections were counted in each preparation.

Localization of ASGP Binding Sites in the PM

Freshly isolated PM sheets ($\sim 2\text{--}5 \text{ mg/ml}$ in 0.25 M sucrose) were fixed by the addition of an equal volume of 3% p-formaldehyde in 0.15 M Na arsenate, pH 7.4, 6 mM CaCl_2 , incubated for 20 min at 4°C , and centrifuged at $10,000 \text{ g}$ for 2 min onto a 2 M sucrose cushion (Beckman microfuge). The prefixed PM was resuspended in 0.25 M sucrose at a protein concentration of $4\text{--}5 \text{ mg/ml}$ for subsequent binding, autoradiographic (ARG) localization and penetration experiments.

In preliminary studies, we found that the BF space was not accessible to particulate tracers. Therefore, in some PM samples we included saponin, a plant glycoside that has been shown to permeabilize plasma membranes (38, 39). Two levels of saponin were used, "low" ($0.05\text{--}0.15 \text{ mg/mg}$ PM protein) and "high" ($0.16\text{--}0.24 \text{ mg/mg}$ PM), because the morphological preservation of PM domains tended to be somewhat variable in the presence of the detergent. Prefixed PM sheets that had never been exposed to saponin were carried through all treatments.

BINDING: The ASGP binding activity present in unfixed PM, prefixed PM, and prefixed, saponin-permeabilized PM was assayed as described above, except that ^{125}I -ASOR bound to PM was separated from free ligand by centrifugation (2 min at $10,000 \text{ g}$) followed by one rinse. The radioactivity remaining in the pellet was measured.

ARG LOCALIZATION: Prefixed PM sheets ($\sim 500 \mu\text{g}$) were incubated in ASGP binding buffer and saturating levels of ^{125}I -ASOR, either in the absence or presence of saponin ("low" and "high"), for 20 min at room temperature with agitation (total volume = 0.4 ml). The suspensions were diluted with 4–5 volumes of the same buffer and centrifuged onto a 2 M sucrose cushion as described above. After resuspension in 0.1 M Na arsenate, the PM sheets were fixed for 20 min at 4°C with an equal volume of 3–4% glutaraldehyde in 0.1 M Na arsenate, pH 7.4, centrifuged as above, and resuspended in 0.25 M sucrose. They were embedded in agarose as described by Zeitlin and Hubbard (29), processed for EM as described above and for EM-ARG according to the method of Salpeter and Bachmann (40) as described earlier (35).

Micrographs of PM present in ARG preparations were taken at a standard magnification of $10,000$ both at random and of selected regions where the membranes were well spread out. Quantitative analysis was performed on prints at $\times 27,000$. The distribution of ARG grains associated with PM sheets was determined by counting grains overlying the following membranes: (a) the bile canalicular domain; (b) the lateral surface and associated vesicles (these two types of membranes were not resolved by our ARG analysis); (c) the sinusoidal domain; (d) unidentified membrane; and (e) rough microsomes and mitochondria. The identification of the three hepatocyte plasmalemmal domains was based on morphological criteria (presented in Results). The relative surface area of each membrane category was determined on the same micrographs using the line intersection method described above.

PENETRATION: It was important to determine in each experiment that ASOR could gain access to all membrane surfaces. Therefore, a series of tubes parallel to that used for the ARG localization, containing prefixed PM with or without saponin ("low" and "high"), was incubated in ASGP binding buffer with the particulate tracer, cationized ferritin, at a final concentration of $\sim 3 \text{ mg/ml}$. Cationized ferritin ($pI = 7.4$) was prepared by the procedure of Danon et al. (41) as modified by Kanwar and Farquhar (42), using 50 mg EDC. After incubation, the PM sheets were rinsed and processed for EM visualization as described above.

RESULTS

In this section, we first describe the isolation of a membrane fraction and demonstrate that it is highly enriched in several

TABLE I
Analysis of Plasma Membrane Fractionation*

Step	5'-Nucleotidase	Protein	Relative enrichment \ddagger
	Percent distribution \pm SD	Percent distribution \pm SD	
1. 280 g 5 min			
Supernate	88 \pm 7	94 \pm 3	<u>0.85 \pm 0.15</u>
Pellet	12 \pm 7	6 \pm 3	
2. 1,500 g 10 min			
Supernate	52 \pm 12	68 \pm 7	
Pellet	48 \pm 11	32 \pm 6	<u>1.0 \pm 0.22</u>
3. Flotation			
Interface	48 \pm 15	1.9 \pm 0.6	<u>22 \pm 6</u>
1.18 g/cm ³ layer	25 \pm 5	26 \pm 3	
Pellet	25 \pm 17	72 \pm 4	
4. 1,500 g 10 min			
Supernate	19 \pm 6	51 \pm 8	
Pellet (PM) \S	81 \pm 6	49 \pm 8	<u>36 \pm 21</u>

* Five separate experiments were analyzed and the results tabulated above. The 5'-nucleotidase activity and protein present in each fraction were measured and their percent distributions within each step calculated, after correction for recovery. Recoveries of enzyme activity and protein at each step were $>91\%$. The underlined fraction in each step constituted the starting material for the subsequent step and was normalized to 100%.

\ddagger Relative enrichment is defined as the ratio of specific 5'-nucleotidase activity in a fraction to the specific activity in the homogenate with the latter normalized to 1. Only relative enrichments for underlined fractions are presented.

\S The yield of total 5'-nucleotidase in the final PM fraction can be calculated from the percentages of recovered activity in each underlined fraction (i.e. $0.88 \times 0.48 \times 0.48 \times 0.81 \times 100 = 16\%$ of initial filtered homogenate 5'-nucleotidase).

PM markers and significantly depleted in biochemical markers for other subcellular organelles. We then present a morphological characterization of this PM fraction.

Isolation of Liver Plasma Membrane

The distribution of 5'-nucleotidase activity, a conventional plasma membrane marker, is presented in Table I to illustrate the distribution of plasma membrane containing this enzyme at each step in the fractionation.

Gentle homogenization of rat livers in buffered sucrose yielded membrane sheets that could be separated from the bulk of contaminating mitochondria and microsomes by a series of differential and isopycnic centrifugations. The first step in our isolation procedure, low-speed centrifugation of the filtered homogenate ($280 \text{ g} \times 5 \text{ min}$), was performed in order to sediment unbroken cells and allow their subsequent homogenization without further rupturing the membrane sheets already formed. The two low-speed centrifugations constituting Step 1 yielded a supernate containing $>85\%$ of all markers assays (e.g., 5'-nucleotidase, Table I). The next step (Step 2), centrifugation at $1,500 \text{ g}$ for 10 min, separated larger structures (e.g., nuclei and membrane sheets) from vesicular and soluble elements. It can be seen from Table I that $\sim 50\%$ of the 5'-nucleotidase in the low-speed supernatant was sedimented at $1,500 \text{ g}$, indicating the presence of a substantial fraction of this enzyme activity in very large structures. In the next step (Step 3), $\sim 50\%$ of the sedimentable enzyme activity from Step 2 was found to be in structures whose density in sucrose was less than 1.18 g/cm^3 since they floated to a $1.04/1.18 \text{ g/cm}^3$ interface.

Furthermore, a significant enrichment (22-fold) of the enzyme was achieved at this step. Finally, >80% of the material collected from the interface during Step 3 could again be sedimented under the same conditions as those used before flotation (i.e., Step 4, 1,500 g min), indicating that extensive vesiculation had not occurred. The final yield of plasma membrane, as measured by 5'-nucleotidase activity, averaged 16%, with $\geq 90\%$ recovery at each step (Table I).

Biochemical Characterization of the Isolated Plasma Membranes

Having established a procedure that routinely yielded $\sim 20\%$ of the activity of a classical plasma membrane marker, we next assessed the purity of the PM preparation by assaying the activities both of enzymes considered to mark different domains of the PM and of enzymes marking other organelles that might contaminate the PM preparation (Table II). Only data for the final PM fraction are presented, but balance sheets were kept on all enzymes assayed and recoveries were 85–105% at each step in the fractionation. Both 5'-nucleotidase and alkaline phosphodiesterase I (APDE I), two PM activities reportedly concentrated in BC membrane (43, 44), were 40-fold enriched in the PM over the starting homogenate, indicating a significant purification of membrane containing these activities in the fraction. These values are among the highest reported for a PM preparation consisting of membrane sheets. An SF marker, the ASGP receptor, was similarly enriched in the final PM (47-fold, Table II), with yields comparable to those of the other

two PM markers (Table II, 16–17% for each). Assays for other enzyme activities used as PM markers, such as leucine aminopeptidase and alkaline phosphatase, revealed lower yields and enrichments of these activities in the final PM (data not shown), in agreement with the findings of others (13, 14).

Contamination of the PM fraction by mitochondria, nuclei, and lysosomes was relatively low, as evidenced by the yield and depletion of the respective marker activities. For instance, only 0.12% of cytochrome oxidase, an inner mitochondrial membrane enzyme complex, was found in the final PM, with a 0.22-fold enrichment relative to the homogenate. Similar values were obtained for monamine oxidase (data not shown), an enzyme present in the outer mitochondrial membrane (45). Membrane derived from the endoplasmic reticulum (ER), as measured by glucose-6-phosphatase and NADH-cytochrome c reductase activities, appeared to be the major organelle contaminant of the PM (Table II). That is, although only 0.3–0.5% of these activities was recovered in the final fraction, there was very little to no depletion of their specific activities in the PM over those in the initial filtered homogenate. Similar results were obtained for NADPH-cytochrome c reductase (data not shown). However, in addition to being ascribed to the ER membrane, all of these enzymes have been ascribed to Golgi membranes (30, 46, 47), the NADH-requiring reductase to outer mitochondrial membranes (47) and even to the plasma membrane of liver (48). Nevertheless, we have taken the one-fold enrichment of these enzymes as indicative of ER contamination. Morphological examination of the PM (to be described below) confirmed this judgement. However, the values we obtained were quite comparable to those found in other studies in which full recoveries were reported (e.g. reference 21).

The quantitative contribution of the Golgi membranes, as assessed by galactosyl and sialyl transferases, has been difficult to determine, due to poor recoveries, particularly at the flotation step (Step #3, $\sim 30\%$ recovery). However, 80–85% of both enzyme activities remained in the supernate after low-speed centrifugation (Step #2, 80% recovery), indicating removal of the bulk of the Golgi membranes before the flotation step.

Morphology of the Isolated Plasma Membranes

IDENTIFICATION OF THE THREE MEMBRANE DOMAINS: Morphologic examination of the PM preparation revealed the presence of extended sheets of membrane (Fig. 1) with many features reminiscent of the hepatocyte plasmalemma *in situ*. The BC and LS membranes were very prominent, the former consisting of small vesicle profiles enclosed within an encircling membrane, and the latter of straight segments of parallel membranes often interconnecting two BC domains. At higher magnification (Fig. 2a), gap junctions and desmosomes were evident along the LS. Short segments of membrane extending from the LS and terminating in free ends could be seen (Fig. 1) and have been described in other PM preparations (e.g. 7, 11, 22). However, our preparation also contained large amounts of membrane that had the following characteristics expected of SF membrane (Figs. 1 and 2): (a) close juxtaposition to *bona fide* hepatocyte plasma membrane (i.e., BC and LS membrane); (b) numerous loose, irregular vesicle profiles, some having the appearance of microvilli; (c) fibrillar material associated with only one side of the membrane; and (d) coated pits (Fig. 2b–d). Identification of membrane with these characteristics as SF was confirmed by examination of serial sections (Fig. 3) in which structures present only in the LS of parenchymal cells of liver (e.g., desmosomes) were in continuity with such membrane. This finding suggested

TABLE II
Biochemical Characterization of the Rat Liver Plasma Membrane Fraction*

Marker†	Yield§		Relative enrichment
	% \pm SD	(#)	\pm SD
Protein	0.4 \pm 0.13	(10)¶	**
5'-Nucleotidase (PM)	17.4 \pm 6	(9)	39 \pm 10
Alkaline phosphodiesterase I (PM)	17.0 \pm 5.6	(4)	40 \pm 9
Asialoglycoprotein binding activity (PM)	16.3 \pm 6	(6)	47 \pm 18
NADH-cytochrome c reductase (ER)	0.3 \pm 0.2	(10)	1 \pm 0.5
Glucose-6-phosphatase (ER)	0.5	(2)	0.8
β -N-acetyl-glucosaminidase (lysosomes)	0.22 \pm 0.13	(3)	0.66 \pm 0.3
Cytochrome oxidase (mitochondria)	0.12 \pm 0.07	(4)	0.22 \pm 0.1
DNA (nuclei)	0.26	(1)	0.3
RNA	0.5	(2)	0.3

* Enzyme and chemical assays were performed on every fraction throughout the isolation. The recoveries at each step were $>85\%$, with the exceptions of those for DNA and RNA after Step 2. Due to the insensitivities of these assays and low amounts present after Step 2, recoveries were more variable (80–120%). Homogenate specific activities were as follows (all given in μ moles product formed per h per mg protein, except cytochrome oxidase): 5'-nucleotidase, 1.6 \pm 1.0; APDE I, 3.1 \pm 1.1; NADH-cytochrome c reductase, 5.7 \pm 2.1; glucose-6-phosphatase, 7.2 (aver.); β -N-acetyl-glucosaminidase, 0.64 \pm 0.44. Cytochrome oxidase, 3.9 \pm 0.5 Δ log OD units per min per mg protein.

† The predominant cellular organelle in which an enzyme activity or chemical is found in liver is noted in parentheses after the marker.

§ The yield (\pm standard deviation, SD) of each marker is the percent of the initial homogenate activity present in the final membrane fraction after correcting for recoveries. See footnote § in Table I for method of calculation.

|| Relative enrichment is defined as the ratio of specific activity in the PM to specific activity in the homogenate with the latter normalized to 1.

¶ Number of separate experiments.

** The yield of protein was 0.77 \pm 0.3 mg per g wet weight of liver.

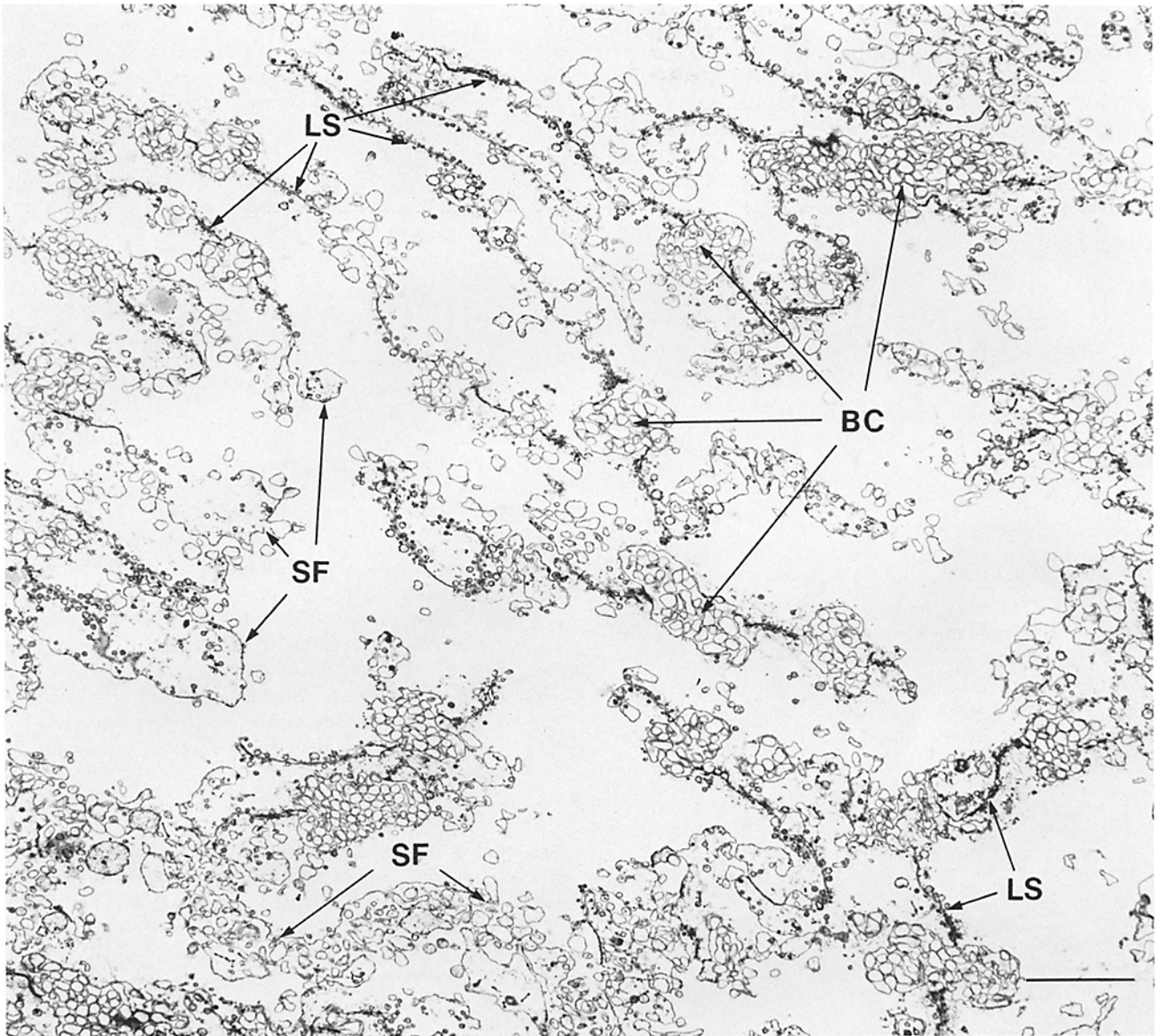


FIGURE 1 Electron micrograph of isolated rat liver plasma preparation. The predominant structures in this preparation are sheets of membrane having three morphologically distinct regions: the bile canicular domain (BC), comprised of vesicle profiles contained within an encircling membrane; the lateral surface domain (LS), characterized by straight segments of two parallel membranes in continuity with the membranes lining bile canaliculi; and the sinusoidal front domain (SF), composed of groups of irregular vesicle profiles, a number of which can be seen to project from a common segment of membrane. Lipid droplets and an occasional mitochondrion are seen in these preparations. Bar, 2 μm . $\times 7,820$.

very strongly that all three hepatocyte plasma membrane domains were represented in our PM preparation.

LOCALIZATION OF ASGP BINDING SITES IN THE PM: The presence and identification of SF membrane in our preparation was further confirmed by an EM autoradiographic approach. It is well established that functional ASGP receptors are present along the sinusoidal surface of hepatocytes *in situ* (1, 28), and we had already determined that our PM preparation was highly enriched in accessible ASGP receptor activity (Table II). Therefore, we asked whether ASGP binding sites were concentrated in membrane previously identified as SF on the basis of morphological criteria. We chose EM-ARG because it required the use of only one molecule, the ^{125}I -labeled ligand, and because it was of sufficient resolution (1,000 Å for ^{125}I [50]) to distinguish among membrane domains of dimensions 1–2 μm . By prefixation with low concentrations of p-formaldehyde (1.5%), the distinctive morphology of the three

membrane domains was maintained throughout the EM-ARG procedure (Fig. 4a) and substantial and specific ASGP binding activity was retained (Table III, PM #2). However, it was necessary to include low concentrations of the detergent saponin to insure access to all membrane surfaces, especially that of the BC domain. Although exposure of prefixed PM to saponin did alter the PM morphology, domains could still be recognized (Fig. 4b–d), and accessibility of all surfaces to molecules the size of ferritin (500 kdaltons) could be demonstrated (Fig. 5). Interestingly, saponin did not increase the amount of ^{125}I -ASOR specifically bound to the prefixed PM (Table III, PM #4), suggesting that previously inaccessible regions (BC) did not contain ASGP binding sites. Table IV presents the distribution of ^{125}I -ASOR binding sites among five membrane categories of PM incubated in the absence (A) or presence (B) of saponin. Of the ARG grains associated with the three recognizable hepatocyte surface domains (70% in A

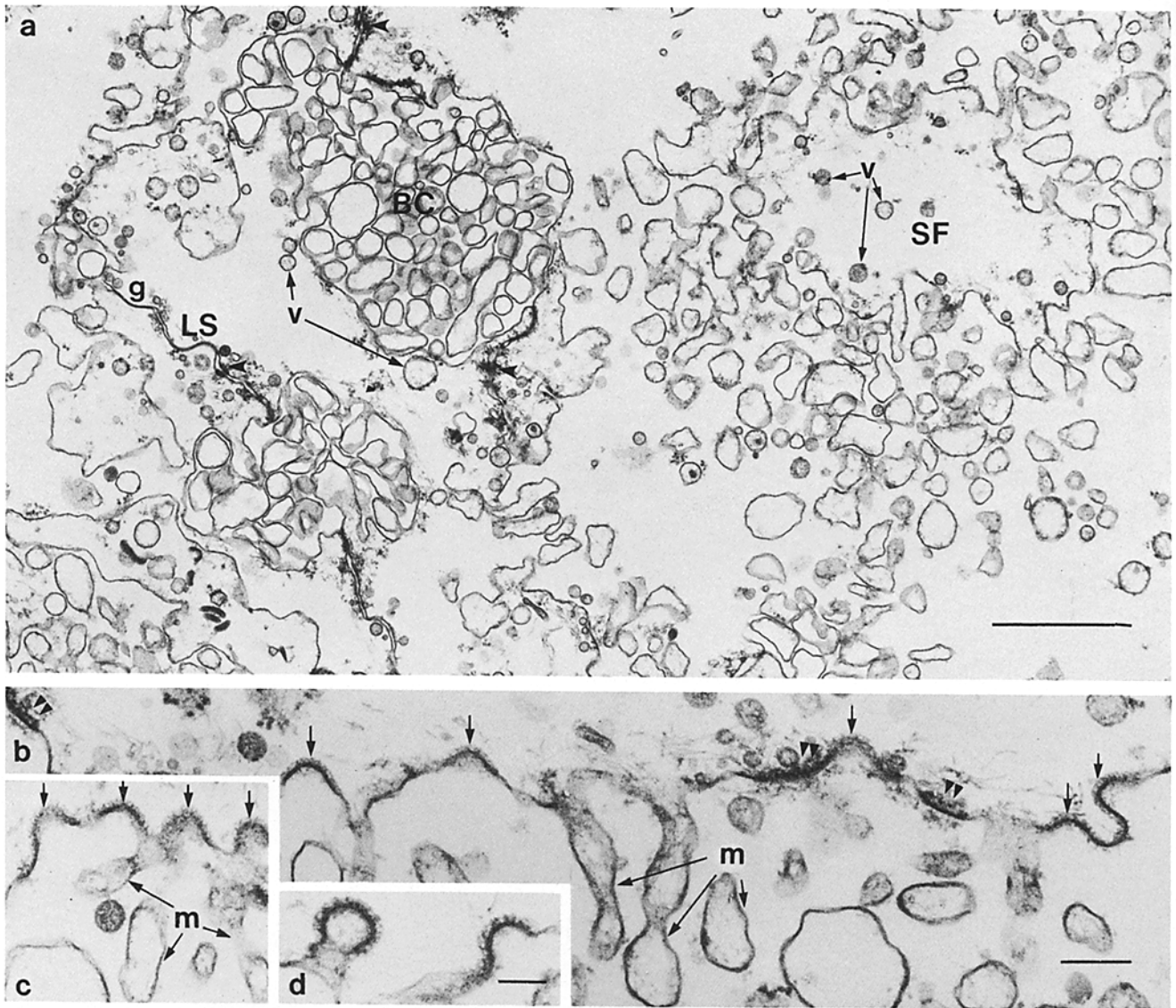
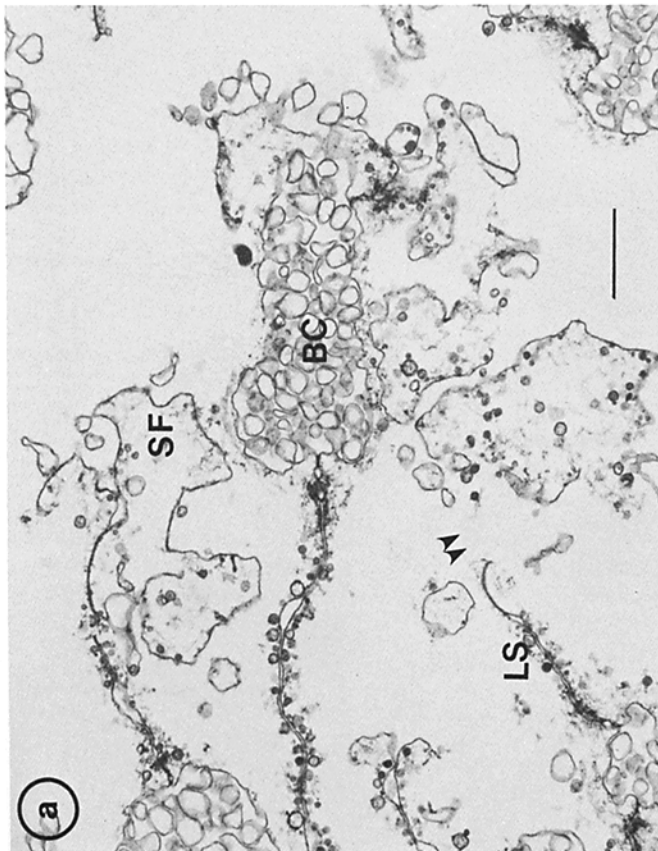
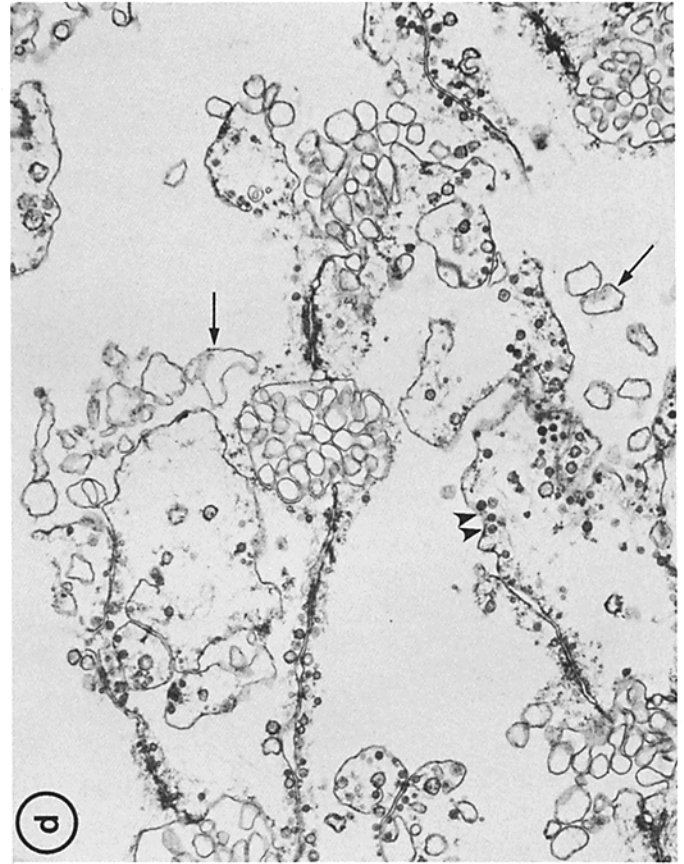
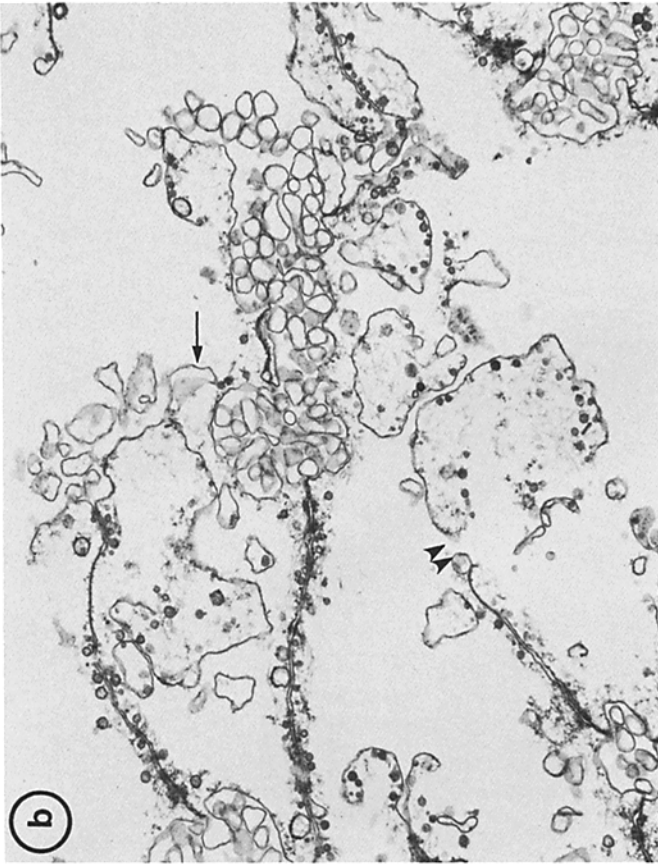


FIGURE 2 Electron micrographs of isolated rat liver plasma membranes. (a) The three morphologically distinguishable domains of the isolated PM are seen at higher magnification than in Fig. 1: bile canalicular (BC), lateral surface (LS), and sinusoidal front (SF). Adherent vesicles (v) are evident, as are several lateral surface specializations: a gap junction (g) and desmosomes (arrowheads). Fibrillar material is associated with both LS and SF membranes. Bar, 1 μm . $\times 20,000$. (b-d) The SF domain. Microvillar projections (m) are in continuity with membrane that has several distinctive features all predominantly on one surface (the cytoplasmic): filaments and small vesicles; bristle-coated regions (arrows) often having the shape of pits (c and d); and regions with densely staining material on the cytoplasmic surface (double arrowheads) and corresponding fibrillar material on the external surface. b: Bar, 0.2 μm ; $\times 51,000$. c and d: Bar, 0.1 μm ; $\times 67,500$.

FIGURE 3 Electron micrographs of serial sections from a rat liver plasma membrane preparation. Micrographs were taken of the same region of a PM pellet in 10 successive sections of which the first, third, fifth, and sixth sections are pictured in a, b, c, and d, respectively. A double arrowhead marks the same location in each section. In d, membrane identified morphologically as SF is continuous with identifiable LS membrane at the double arrowhead. However, in a-c, there is not continuity. One irregular membrane profile (marked by an arrow in b, c, and d) is connected to SF membrane only in b, not c or d. Other such profiles are closely associated with each other and with membranes that are in continuity with LS but appear in most sections to be distinct structures. These are SF membrane projections. Structural continuity of the three domains can readily be followed in the four panels and emphasizes the large size of the PM sheets isolated, each of which represents major portions of the surfaces of several cells. Bar, 1 μm . $\times 12,000$.



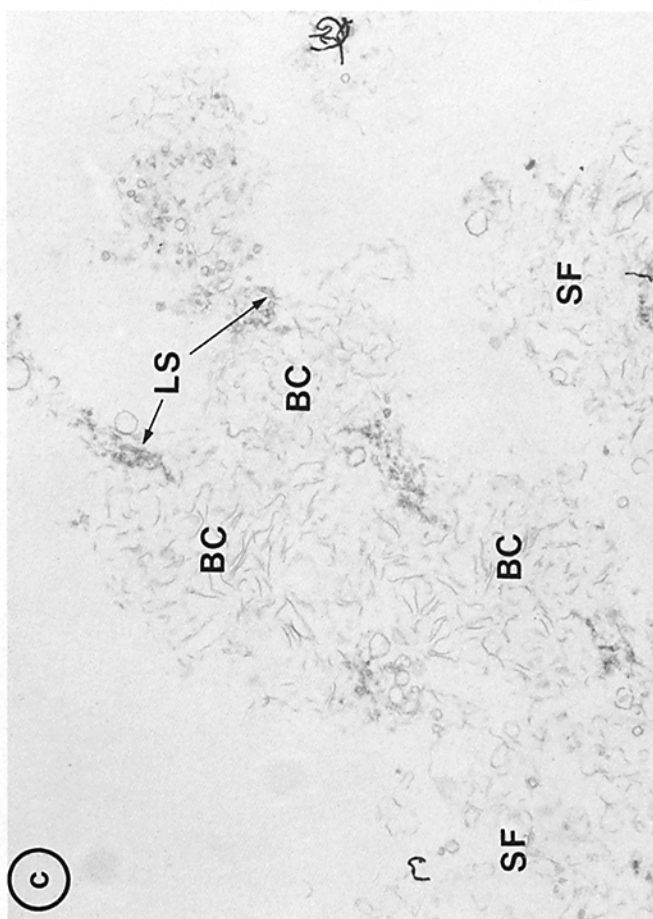
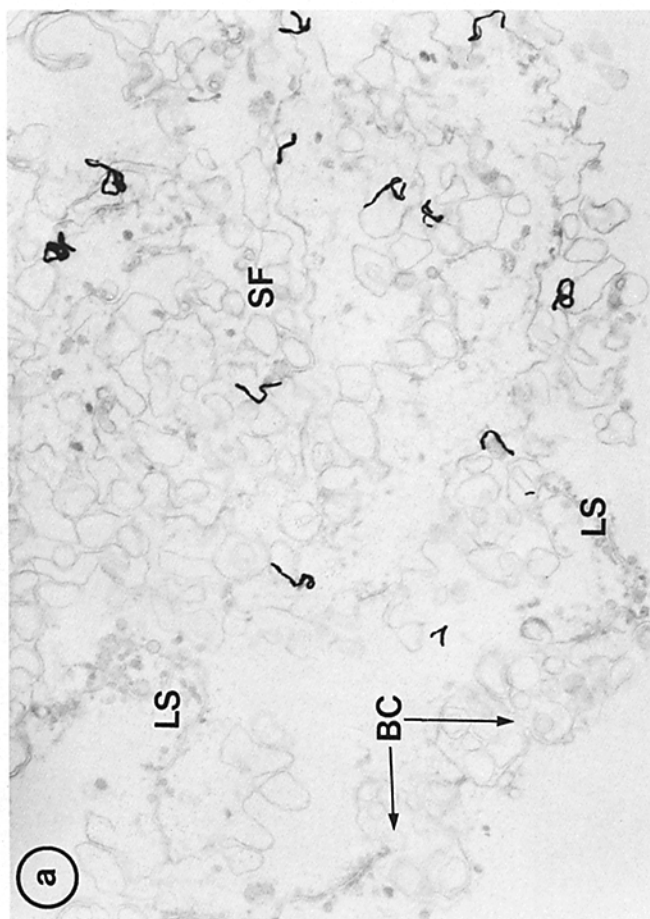
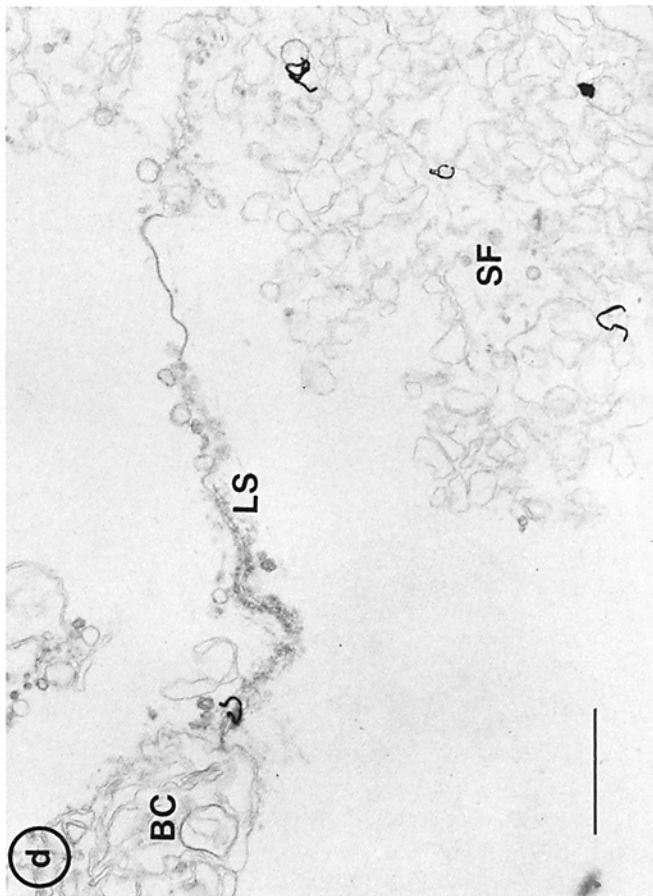
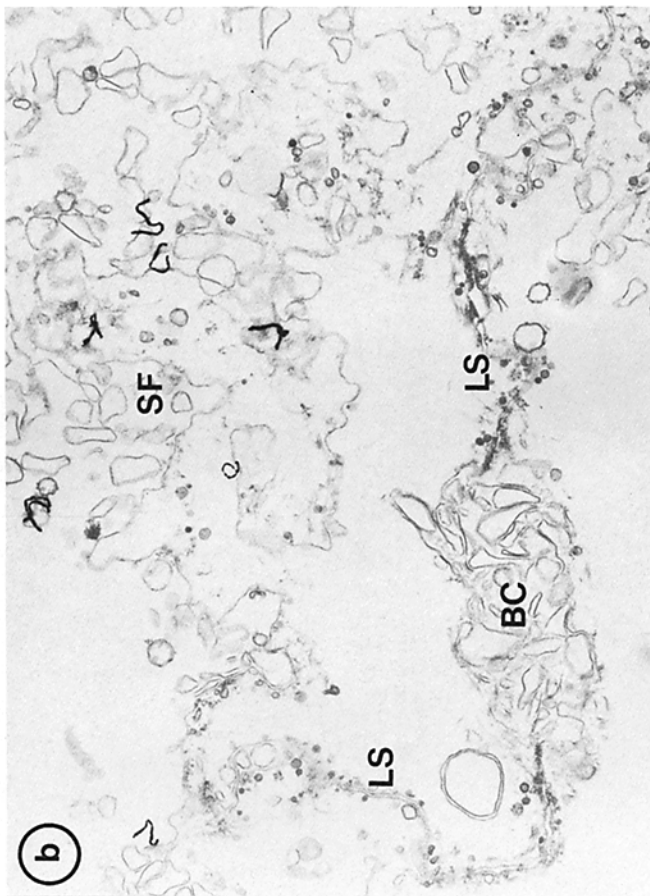


TABLE III
Binding of ^{125}I -Asialo-orosomucoid to Isolated Plasma Membranes Used for EM-ARG

Membrane preparation*	Competitor‡	Saponin§		Amount of ^{125}I -ASOR bound
		mg/mg PM protein	$\mu\text{g}/\text{mg}$ PM protein	
1. 46 μg , unfixed	—	—	—	0.33
46 μg , unfixed	40 μg ASOR	—	—	0.006
46 μg , unfixed	40 μg OR	—	—	0.30
2. 65 μg , unfixed	—	—	—	0.38
65 μg , prefixed	—	—	—	0.22
3. 50 μg , prefixed	—	—	—	0.2
50 μg , prefixed	50 μg ASOR	—	—	0.009
50 μg , prefixed	50 μg ASOR	0.24	—	0.009
4. 100 μg , prefixed	—	—	—	0.14
100 μg , prefixed	—	0.15	—	0.14
100 μg , prefixed	—	0.24	—	0.12

* The ASGP binding activity in four different PM preparations was assayed before and after prefixation in p-formaldehyde as described in Methods.

‡ ~ 200-fold excess of unlabeled ASOR or the fully sialylated OR were added to the incubation mixture to assess the specificity of ^{125}I -ASOR binding.

§ Saponin was added to the incubation mixture at the final ratios indicated in either the absence (PM #4) or presence (PM #3) of excess unlabeled ASOR to assess saponin's effect on the extent and specificity of ^{125}I -ASOR binding.

|| In three separate PM preparations, the average binding activity retained after prefixation was 77%, with a range from 58% (presented here) to 100%.

and 53% in *B*), >90% were localized to SF or LS membranes. Furthermore, membrane that we had characterized as SF exhibited a 10-fold higher grain density (a measure of binding sites per unit length (or area) of membrane) than did the BC surface, whether or not saponin was present. Similar quantitative results were obtained at "low" saponin levels where the domain morphology was better preserved than at "high" saponin levels (Fig. 4*b* and *d*). The EM-ARG results not only confirmed the identification of SF membrane, but revealed that ASGP binding sites were largely absent from BC membrane. Two additional points should be made. First, ARG grains were found over LS membrane, but the grain density was lower than that in the SF (0.69 vs. 2.12 in Table IV, *A*). One explanation is that vesicles closely associated with the LS (within 1,000 Å) do not contain binding sites but were counted in surface area measurements. Secondly, a fraction (30–46%) of the ^{125}I -ASOR was localized to "unidentified membrane" in these PM preparations, much of which is probably SF.

MORPHOMETRIC ANALYSIS OF THE PM: Having established both biochemically and morphologically that all three membrane domains were present in our PM preparation, we next used morphometry to quantitate the amount of each represented. The results of six separate experiments are presented in line *A* of Table V together with the relative enrichment of 5'-nucleotidase for five PM preparations analyzed.

First, the total amount of membrane identified as plasmalemmal (BC + LS + SF) was large ($77 \pm 4\%$) and varied little among five preparations (73–81%). However, the relative enrichments in 5'-nucleotidase in the same preparations ranged from 15- to 41-fold ($31 \pm 12\times$). This result suggested that low relative enrichments of the biochemical marker were due to contamination by nonmembranous protein. Second, the rather large fraction of unidentified membrane (line *A*, $23 \pm 4\%$, Table V) undoubtedly reflected inclusion of some SF membrane in this category. Rough microsomes also contributed to this category, and their presence was apparent both biochemically (Table II) and morphologically (Fig. 2). However, few mitochondria, Golgi membranes or lysosomes were evident (<2% each), confirming the biochemical results described above.

When we compared the average distribution of the plasma membrane domains in our PM preparation (line *B*, Table V) to that reported by Weibel (6) for hepatocytes *in situ* (line *C*, Table V), we found that there was relatively more BC membrane in the isolated PM (line *B*, 30% vs. line *C*, 12%). SF membrane represented a substantial portion of the identifiable plasma membrane in the isolated PM (line *B*, 61%, Table V), but LS membrane was under-represented when compared to the data obtained *in situ* (line *B*, 9% vs. line *C*, 16.8%).

FILAMENTS ARE PRESENT IN THE PM: In addition to the presence of all three membrane domains, a striking feature of the PM preparation was the abundance of filaments, some intimately associated with desmosomes (Figs. 2*a* and 3) and others forming a filamentous network along the cytoplasmic aspect of the SF membrane (Fig. 2). Although filaments were associated with the basal portion of the BC membrane, they were largely absent from the microvillar projections that appeared as vesicle profiles within a BC domain (e.g. Figs. 2*a* and 3).

Numerous small vesicles, some with ribosomes attached, appeared to be trapped by the filaments associated with the PM, particularly along the LS membrane (e.g. Figs. 2 and 3). In the hope of reducing microsomal contamination, we sought conditions that would selectively remove the filaments, while at the same time maintaining the morphological integrity (and identity) of the three plasma membrane domains and the activities of our enzyme markers. Although we did not completely eliminate the problem of microsomes in our PM preparation, we have established conditions that disassemble PM-associated filaments and yield a highly enriched PM that is morphologically intact. Furthermore, we have identified the filaments and their molecular components. These results are presented in the following paper (15).

DISCUSSION

In the present study we have described the isolation and characterization of a rat hepatocyte plasma membrane prepa-

FIGURE 4 Electron microscope autoradiographs of prefixed PM incubated with ^{125}I -ASOR. PM was prefixed, incubated with ^{125}I -ASOR in the absence (a) or presence (b–d) of saponin and processed for EM-ARG. (a) Control PM. The three membrane domains are clearly recognized (labeled BC, LS, and SF) and ARG grains overlie only the SF domain. (b and d) Low saponin PM (0.14 mg/ml): two different PM preparations, exposed to the low amount of detergent, exhibit a similar morphology. All domains are still distinct, with the BC set off by the junctional complexes and dense filamentous regions along the LS. Several ARG grains overlie the SF domain. (c) High saponin PM (0.24 mg/mg). Although the morphology of PM in this condition is dramatically altered, BC can still be identified as regions bound on both sides by dense filamentous areas and the LS by gap junctions and desmosomes. The SF is identified as large membrane regions not having the characteristics of BS or LS. The PMs in a and c are from the EM-ARG experiments, A and B, summarized in Table IV. Bar, 1 μm . $\times 17,500$.

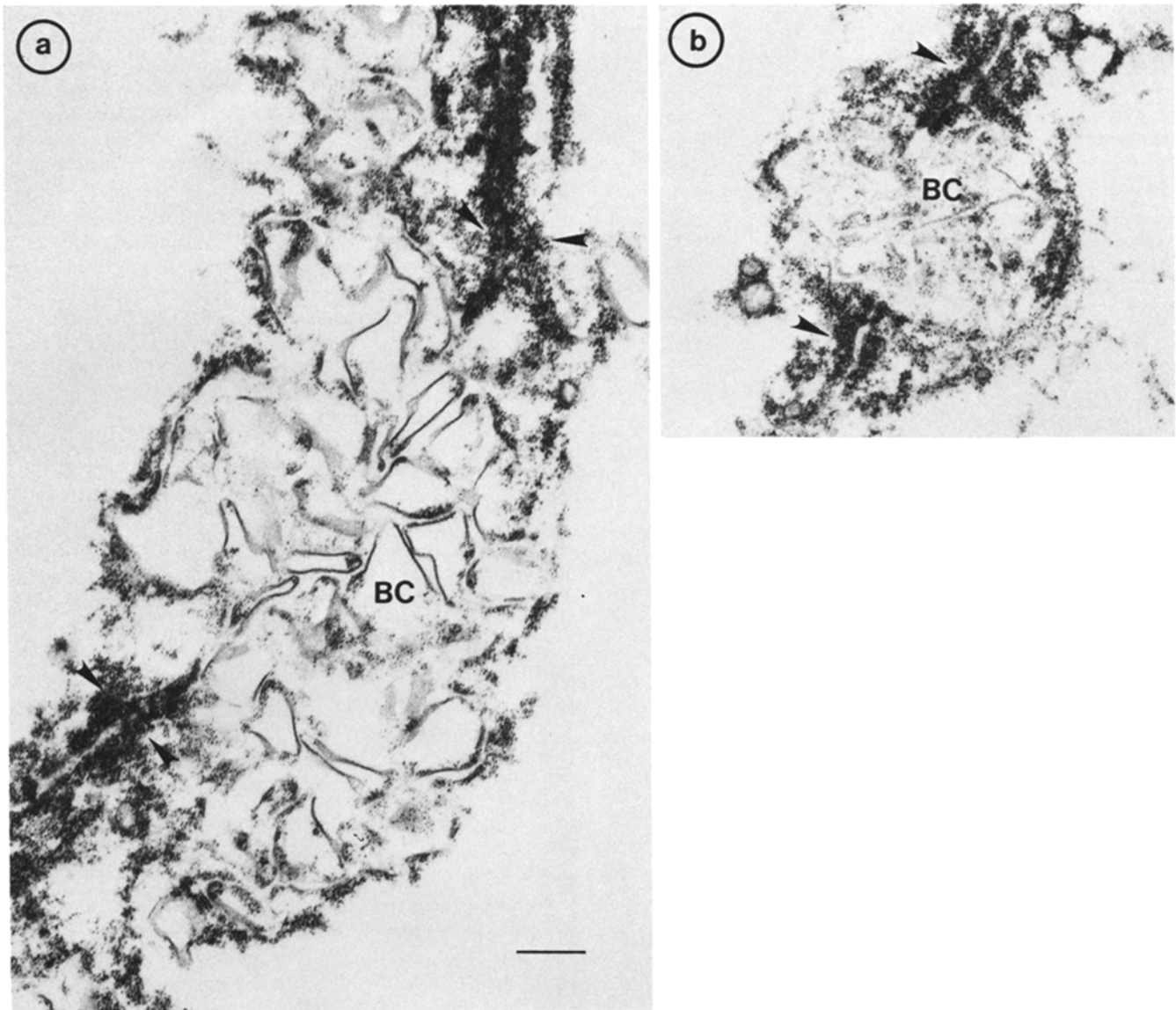


FIGURE 5 Penetration of the bile canaliculus by cationized ferritin. Prefixed PM (same experiment as that presented in Fig. 4 a-c and Table IV) was incubated with cationized ferritin in the presence of low (a) or high (b) saponin. (a) Low saponin PM (0.14 mg/ml). Small amounts of ferritin are seen within the BC. (b) High saponin PM (0.24 mg/ml). Penetration of the BC by ferritin is clearly evident. LS (arrowheads) is readily identified by the heavy deposition of ferritin onto filaments along the LS. Bar, 0.2 μ m. \times 50,000.

ration that contains all three major membrane domains of this epithelial cell in substantial amounts. To our knowledge, this is the first such report.

The Plasma Membrane Isolation Procedure

The isolation procedure we adopted was modified extensively from a method previously published (13). Our procedure offers several advantages: (a) it is fast, taking a total of 4-5 h; (b) it is simple, requiring four low-speed centrifugations and a single flotation through a one-step sucrose gradient; and (c) it is effective, with routine yields of 12-20% and relative enrichments of PM markers in the range of 30- to 50-fold.

Several features of the isolation procedures were found to be essential to successful preparation of highly-purified PM. (a) To reduce aggregation of subcellular organelles by glycogen, we had to starve rats for at least 18 h before sacrifice. (b) To avoid the increased amounts of connective tissue and PM-associated filaments found in livers of older rats, we used livers

from young rats (120 g). (c) To avoid autolysis, we homogenized each perfused liver within 5 min of excision. (d) To reduce vesiculation and/or production of small membrane fragments, we used very gentle homogenization, with minimal generation of bubbles or vacuum during either the up or down strokes. (e) To reduce contamination by ER membranes, we found it helpful to resuspend the 1,500 g pellet in preparation for flotation in a volume twice that of the initial homogenate volume.

Characterization of the Isolated Plasma Membrane

USE OF BIOCHEMICAL MARKERS: One approach to following and subsequently characterizing a subcellular fraction has been to use "markers," enzyme activities or macromolecules that are purportedly specific to cellular components, usually so demonstrated by cytochemistry or the marker's co-enrichment with a morphologically recognizable cellular com-

TABLE IV
EM-ARG Distribution of ¹²⁵I-ASOR in Isolated Plasma Membranes *

Parameter	Membrane Category				
	A. No Saponin Treatment				
	Bile canalicular domain	Lateral surface and associated vesicles	Sinusoidal domain	Unidentified‡	Rough micro-somes and mitochondria
ARG grains (percent of total)	5%	11%	55%	28%	1%
Membrane surface area (percent of total)	20%	16%§	26%	34%	4%
Relative grain density	0.25	0.69	2.12	0.82	0.25
	B. High Saponin Treatment¶				
ARG grains (percent of total)	2%	6%	46%	45%	1%
Membrane surface area (percent of total)	15%	16%	27%	46%	4%
Relative grain density	0.16	0.70	1.68	0.98	0.25

* The results presented are from one EM-ARG experiment. The analysis was performed as described in the Methods on micrographs taken of selected regions of the PM pellet, where membrane sheets were separated enough to allow domain identification. Total grains counted: Experiment A, 343; Experiment B, 211. Analysis of regions photographed at random yielded comparable results, but fewer total grains were counted.

‡ The large size of the unidentified membrane category in the PM of both experimental conditions reflects the difficulty in identifying SF membrane as such if it is not in obvious association with LS. In both prefixed and high saponin-permeabilized PM, the SF domain is quite difficult to identify, hence the larger percent of total membrane in the unidentified category as compared to that in conventionally-fixed PM, whose morphometry is presented in Table V.

§ The percent contributed to this compartment by associated vesicles is ~50%. Therefore, ~8% is lateral surface, in agreement with results of Table V.

|| Relative grain density is calculated by dividing % grains by % surface area in each compartment.

¶ A saponin/PM ratio of 0.24 mg/mg PM protein was used in this experiment and a representative field is presented in Fig. 4 c. Fig. 5 b illustrates the bile canalicular region at higher magnification.

TABLE V
Morphometric Analysis of Plasma Membrane Domains in Isolated Membranes and Intact Hepatocytes

Preparation	5'-Nucleotidase		Membrane category			
A. PM*	RSA‡	BC	LS	SF	Other§	
1	15x	35%	7%	39%	19%	
2	20x	26%	7%	41%	26%	
3	40x	12%	5%	56%	27%	
4	39x	16%	6%	59%	19%	
5	41x	26%	9%	41%	25%	
Average (± SD)	31 ± 12x	23 ± 9%	6.8 ± 1.6%	47.3 ± 9%	23 ± 4%	
6	—	22%	5%	52%	22%	
B. PM¶	—	30%	9%	61%	—	
(Relative surface area**)	—	(3.3)	(1)	(6.8)	—	
C. Intact Hepatocytes‡‡	—	12%	16%	72%	—	
(Relative surface area**)	—	(0.75)	(1)	(4.5)	—	

* See Material and Methods for method of analysis used on PM fractions isolated in this study.

‡ RSA, relative specific activity (relative enrichment) of 5'-nucleotidase in the PM preparation.

§ This category includes microsomes (rough and smooth) as the predominant structures, very few (~1%) contaminating mitochondria, as well as membrane likely to be sinusoidal but not in obvious association with the lateral surface membrane (perhaps 20% of this category).

|| This PM preparation was fixed and embedded in agarose such that the membrane sheets were well separated and, therefore, all PM in the preparation could be analyzed.

¶ Average percent distributions of only the three plasma membrane domains in the PM preparations.

** The percent of surface area in each membrane category was normalized to that in the lateral surface.

‡‡ Values for hepatocyte plasma membrane surface area *in vivo* were obtained from reference 6.

ponent. In recent years, the concept of unique "markers," particularly for organelles of the secretory pathway (i.e., RER→Golgi→PM), and perhaps also the endocytic pathway, has been re-evaluated (e.g. 46, 50-52). The evidence so far suggests that overlap of markers among those compartments in temporal continuity with one another may occur, but the extent of the overlap is not currently established.

There are additional uncertainties in the assignment and use of markers for the plasma membrane of polarized epithelial cells, such as hepatocytes. First, the functional heterogeneity of the plasma membrane (i.e., regional domains) necessitates the use of different PM markers for the different domains. Second, there is some evidence for the existence of internal (recycling?) pools of PM enzymes (50, 53). Third, the biochemical assays for PM markers (e.g. 5'-nucleotidase, several ATPases, leucine aminopeptidase, and alkaline phosphatase) may not be specific

for one enzyme in one location and are usually quite different from the cytochemical assays used to localize a particular activity *in situ*.

Conventional markers for the liver plasma membrane have been 5'-nucleotidase (e.g. 14), alkaline phosphodiesterase I (21), and various ATPases (Mg²⁺, 14; Na⁺, K⁺, 13). The localization of several of these activities *in situ* is controversial or uncertain. For instance, histochemical 5'-nucleotidase at the light microscopic level was reportedly concentrated at the bile canalculus (43, 54), but EM cytochemistry revealed its presence in the other two surface domains (e.g. 55). Recently, Na⁺, K⁺-ATPase was localized to the sinusoidal surface of hepatocytes *in situ* (56), which makes this enzyme a potentially useful marker for the SF domain. In fact, others (e.g. 13) have used a biochemical assay for Na⁺, K⁺-ATPase as a PM marker that is purportedly more reliable than 5'-nucleotidase. However,

this assay gave poor recoveries in our hands, largely resulting from a high background of ATPase activities elsewhere in the cell and from the low binding affinity of the specific Na^+ , K^+ -ATPase inhibitor, ouabain, for the rodent enzyme. Therefore, we chose both 5'-nucleotidase and APDE I as BC markers, and accessible asialo-glycoprotein binding activity as a SF (+ perhaps LS) marker, acknowledging the current uncertainties of these biochemical "markers". All of the activities distributed in a similar fashion throughout the fractionation and all were significantly enriched in the final PM. Both the yields and relative enrichments of the two traditional markers in this multi-domain PM preparation were quite comparable to the best reported by others for similar types of PM preparations (i.e., where membrane sheets were isolated), whether sucrose or bicarbonate was used in the initial homogenization medium (8, 12, 13).

USE OF MORPHOLOGY: In addition to the use of conventional biochemical markers to identify and quantitate the various membranes in our isolated PM, we used morphologic criteria to identify the three domains and then used morphometry to quantitate each. SF membrane represents a major fraction of the total in our isolated PM preparation. The accessible ASGP binding activity validates this finding biochemically. The morphometric analysis further indicated that our PM preparation is not precisely representative of the hepatocyte plasmalemma *in situ*, because the BC membrane domain is over-represented. Nonetheless, this is to our knowledge the first documented report of substantial amounts of *bona fide* SF membrane isolated in continuity with the other two domains.

Others have reported the isolation of SF-enriched preparations from either a starting microsomal fraction (14, 21) or PM sheets subsequently vesiculated and separated from BC vesicles on sucrose gradients (e.g. 9, 13). There are several difficulties in interpreting the results of these other studies. First, documentation was based on use of biochemical markers (e.g. glucagon-stimulated adenylate cyclase, reference 14) whose unique location at the SF has *not* been demonstrated. Second, no morphological criteria were (or could be) used to identify the origin of the vesicles in the final preparations. Finally, although enrichment of several putative SF markers was apparent in the preparations, the presence of substantial amounts of putative BC markers indicated that preparations were not composed solely of membrane derived from one domain. In fact, in those studies in which PM sheets were vesiculated, it is possible that the "SF" subfractions were derived from the lateral surface. Aside from the obvious surface specializations that form the lateral junctional complexes and (among other functions) prevent free intermixing of bile excretion products and plasma, the lateral surface is in physical continuity with the sinusoidal front and may be functionally equivalent. There is suggestive evidence for this hypothesis, because electron microscopic (EM) tracers have free access up to the level of the tight junction and several receptors (integral membrane components) have been reported to be present along both the lateral and sinusoidal surface (57). Definitive proof must await isolation of these two domains. It is our eventual goal to separate all three membrane domains and to characterize them. We think this PM preparation is ideal starting material.

ASSESSMENT OF CONTAMINANTS: The level of contaminating organelles in our PM preparation was quite low, except for the ER. Neither lysosomes nor mitochondria together constituted >6% of the protein in the PM. That is, from the relative enrichments of their marker enzymes given in Table II and

information from the literature regarding the fraction of total liver protein each represents, we can calculate the amount of mitochondrial and lysosomal protein in the final PM, which was 4.6% and 1.5% respectively.³ These values are quite comparable to those reported by others, where full recovery of activities in the other fractions was documented (e.g. 21).

Microsomes derived from the ER were the major contaminating membranes in our PM preparation. We recovered a small amount of two ER markers, whose relative enrichments (the key value) were ~1. Considering that ER is ~20% of total liver protein (58), this means that the PM remained ~20% contaminated by ER. Others have reported 0.5–0.7 × enrichments of glucose-6-phosphatase in their final PM fractions (e.g. 13, 21), indicating 10–15% contamination. In several cases where no activity was detected in the final PM (e.g. 8, 22), full recovery of the enzyme activity in other fractions was not documented. Therefore, our PM fraction is comparable to those of others in this regard. The morphometric analysis confirms that small vesicles (some undoubtedly derived from the ER, due to presence of attached ribosomes) remain a problem. Our efforts to reduce their presence by selective disassembly of the filamentous network within which they appeared trapped has to date been unsuccessful. Their continued association with PM sheets may have a functional significance that at present is not clear.

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³ Taking the example of mitochondria, the maximum theoretical enrichment of a marker would be 5 (100% activity ÷ 20% of cell protein). To obtain the mg mitochondrial protein in our PM preparation, 0.23 units of mitochondrial marker per mg fraction protein ÷ 5 units per mg pure mitochondrial protein × 100 = 4.6%.

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