

# A Domain-specific Marker for the Hepatocyte Plasma Membrane. II. Ultrastructural Localization of Leucine Aminopeptidase to the Bile Canalicular Domain of Isolated Rat Liver Plasma Membranes

LAURA M. ROMAN\* and ANN L. HUBBARD†

\*Section of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; and

†Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

**ABSTRACT** Leucine aminopeptidase (LAP) is an integral membrane glycoprotein localized to the apical membrane domain of intestinal and kidney epithelial cells. By indirect immunofluorescence, we have shown that antibodies raised against rat intestinal LAP recognized a similar protein concentrated in the bile canalicular (BC) domain of the hepatocyte in situ (Roman, L. M., and A. L. Hubbard, 1983, *J. Cell Biol.*, 96:1548–1558). We have extended this localization to the ultrastructural level. When a saponin-permeabilized, agarose-embedded plasma membrane (PM) fraction was incubated with affinity-purified anti-LAP, 85% of the protein A-gold particles associated with the three recognizable PM domains were present in the BC. The levels of labeling on the other two domains (sinusoidal and lateral) did not exceed that observed with nonimmune controls. The concentration of LAP in the BC domain in isolated PM sheets prompted us to use this antigen for the affinity isolation of BC membrane (Roman, L. M., and A. L. Hubbard, 1984, *J. Cell Biol.*, 98:1497–1504, companion paper).

The hepatocyte is a polarized epithelial cell whose plasma membrane is differentiated into three morphologically and functionally distinct domains. However, the extent to which functional differences among the sinusoidal, lateral, and bile canalicular domains reflect compositional differences has not been fully established since successful purification and subsequent characterization of the three membrane regions have not yet been achieved.

Indirect immunofluorescence and subcellular fractionation have been used to demonstrate compositional differences between the apical and basolateral plasmalemmal domains of kidney and intestinal epithelial cells. For example, alkaline phosphatase, sucrase-isomaltase, and leucine aminopeptidase (LAP)<sup>1</sup> have been localized to the apical brush borders of these cells (1–5) and Na<sup>+</sup>, K<sup>+</sup>-ATPase to the basolateral surface (6, 7). Until recently, successful isolation of the do-

main of the hepatocyte plasma membrane has been complicated by a lack of definitive markers for the plasmalemmal domains. In our earlier immunofluorescence study we demonstrated that LAP was concentrated in the bile canalicular (apical) domain of the hepatocyte (8). Furthermore, there was no detectable staining of the sinusoidal or lateral surfaces or of intracellular organelles. Thus, the antigen LAP seemed to be an appropriate marker for the bile canalicular (BC) domain.

As a necessary prelude to the use of LAP in the isolation of BC membrane, we have confirmed the localization of this antigen at the ultrastructural level. We chose isolated plasma membranes (PM) for these localization studies because (a) immunofluorescence results indicated that the predominant site of the LAP antigen was at the plasma membrane, (b) all three major membrane domains (bile canalicular, lateral, and sinusoidal) were present in our plasma membrane preparation and were readily identified by their morphological appearance, and (c) this PM fraction would constitute the starting material for isolation of bile front membrane using LAP as a

<sup>1</sup> Abbreviations used in this paper: BC, bile canalicular (domain); LAP, leucine aminopeptidase; PA-Au, protein A adsorbed to colloidal gold; PM, plasma membrane.

specific marker (see accompanying paper, reference 28). Affinity-purified anti-LAP antibodies and protein A adsorbed to colloidal gold (PA-Au) were used to visualize the distribution of the antigen in agarose-embedded PM sheets.

We found that there was little specific labeling of any of the membrane domains with PA-Au in the absence of added permeabilizing agents. However, in the presence of 0.005–0.05% saponin and affinity-purified anti-LAP, a 20-fold increase in labeling was observed and >85% of all the colloidal gold particles associated with the three recognizable domains were present in the BC. Furthermore, the antigen was present throughout the BC domain. The level of labeling found on the other two domains did not exceed those observed with nonimmune controls. These results indicate that LAP is a useful bile front marker.

Portions of this work have been presented elsewhere (9).

## MATERIALS AND METHODS

### Materials

Reagents were obtained from the following sources: protein A, Pharmacia Fine Chemicals, Piscataway, NJ; chloramine T, Eastman Kodak Co., Rochester, NY; carrier-free Na<sup>125</sup>I, Amersham Corp., Arlington Height, IL; sodium borohydride, saponin, low-gelling agarose type VII, polyethylene glycol (*M*<sub>w</sub> 20,000) Sigma Chemical Co., St. Louis, MO; BSA type V, Miles Laboratories Inc., Research Products Division, Elkhart, IN; paraformaldehyde, gold chloride, Fisher Chemical Co., Fairlawn, NJ; uranyl acetate, DNP-30, nadic methyl-anhydride, dodecylsuccinic anhydride, Poly/Bed 812, Polyscience Inc., Warrington, PA; glutaraldehyde and osmium tetroxide, Electron Microscopy Sciences, Fort Washington, PA. All other chemicals were reagent grade.

### Antibodies

Guinea pig anti-intestinal LAP was prepared and characterized as previously described (8). LAP-specific antibodies were isolated from whole serum using a LAP-Sepharose column (8). Nonimmune guinea pig IgG was isolated by sodium sulfate precipitation and ion exchange chromatography as described earlier (8).

### Protein A-Colloidal Gold

Colloidal gold was prepared by the reduction of gold chloride with 2.5 mM sodium borohydride in 1 mM potassium carbonate, pH 9, according to the procedure of Rash et al. (10). Protein A (850 μg in phosphate buffer, pH 6.1) and a trace amount of <sup>125</sup>I-protein A (1 × 10<sup>7</sup> cpm) were added dropwise to the gold colloid, and the mixture was stirred for 5 min (final pH ~6.2). After stabilization of PA-Au with 0.2% polyethylene glycol, the particles were sedimented at 144,000 g for 30 min at 4°C. The pellets were resuspended with PBS ~4 ml total and applied to two, 35-ml 10–30% glycerol gradients (vol/vol). After centrifugation at 82,000 g (2 h, 4°C), the dark reddish band immediately below the loading zone was removed (20 ml) and used for the labeling studies to be described. Aliquots (2 ml) of the fractionated PA-Au were dialyzed 12–16 h at 4°C against 2 liters of PBS prior to their use in the immunolabeling studies. Two preparations of PA-Au were used for these studies; their mean

size was 4.4 ± 1.3 nm as determined by electron microscopy and comparison to a calibrated grid (Ernest F. Fullam, Inc., Schenectady, NY).

## Isolation of Plasma Membranes

Rat liver plasma membranes (5–7 mg/ml) were prepared according to Hubbard et al. (11). Only freshly prepared membranes were used for the localization studies.

## Fixation and Agarose Embedding of Plasma Membrane Sheets

Aliquots of plasma membranes (0.4 ml) were mixed with 0.25 vol of ice-cold fixative (10% formaldehyde and 0.5% glutaraldehyde and 0.1 M Na phosphate, pH 7.4, final concentrations 2.0%, 0.1%, and 0.02 M, respectively). The membranes were fixed 30 min on ice and then embedded in agarose (Method I). In some experiments, 0.25% saponin was added to the 5 × fixative (final concentration, 0.05%, Method II). Fixed plasma membranes were embedded in agarose as described by deCamilli et al. (12) with minor modifications.

## Immunolabeling of Agarose-embedded Plasma Membranes

The following protocol was modified from that originally published by deCamilli et al. (12). All incubations prior to en bloc staining with uranyl acetate were carried out at 4°C on an orbital shaker (medium speed, Bellco, Vineland, NJ). The antibody and PA-Au solutions were centrifuged for 2 min in an Eppendorf microfuge before use, and all other solutions were filtered through 0.22 μm nitrocellulose (Millipore, Bedford, MA) before their addition to the agarose squares. The details of the protocol we followed are outlined below. Step 1: The embedded samples were divided into aliquots and treated in one of the following ways (see Table I): (A) No saponin: The samples were incubated for 45–60 min in solution A (20 mM Na phosphate, pH 7.4, 0.15 M NaCl, 5 mg/ml BSA). (B) High saponin: The samples were incubated for 45–60 min in solution A containing 0.02% saponin and detergent was included in all steps up to the final fixation in aldehyde. (C) Low saponin: The samples were incubated for 45–60 min in solution A containing 0.005% saponin and detergent was omitted in all subsequent steps. (D) Saponin-fix: Saponin-fixed membranes (Method II) were incubated for 45–60 min in solution A (no saponin) and treated as for aliquot A (no saponin) in all subsequent steps. Step 2: The embedded membranes were incubated overnight in 0.5 ml of solution A saponin containing affinity-purified anti-LAP antibodies or nonimmune IgG (12–20 μg). Step 3: The squares were washed four times for 15 min each in solution A saponin (1 ml). Step 4: The samples were incubated for 4 h in 0.5 ml of dialyzed PA-Au in solution A saponin. Step 5: The squares were washed as described in Step 4. Step 6: The samples were fixed for 30 min in Karnovsky's fixative minus calcium (1.5% glutaraldehyde, 2.5% formaldehyde in 0.1 M cacodylate buffer, pH 7.4). Further fixation of the samples in 1% OsO<sub>4</sub>, en bloc staining, and dehydration were as described by Hubbard et al. (13).

## Effect of Fixation and Saponin Treatment of LAP Activity

The extent to which fixation and saponin treatment reduced or released detectable LAP from the plasma membrane sheets was determined by measur-

TABLE I  
Fixation-Permeabilization Conditions Used for the Localization of LAP in Isolated PM\*

Treatment	Fixation	Permeabilization	Additional treatments
A	2% FA <sup>†</sup> 0.1% GA	None	None
B (High saponin)	2% FA 0.1% GA	0.02% saponin in equilibrium buffer <sup>‡</sup>	Saponin included in all incubations and washes
C (Low saponin)	2% FA 0.1% GA	0.005% saponin in equilibrium buffer	Saponin omitted from all subsequent steps
D (Simultaneous)		2% FA 0.1% GA 0.05% Saponin	Saponin omitted from all subsequent steps

\* See Materials and Methods for further details.

<sup>†</sup> FA, formaldehyde, freshly prepared from para-formaldehyde; GA, glutaraldehyde.

<sup>‡</sup> Equilibrium buffer = 20 mM Na phosphate, pH 7.4, 0.15 M NaCl, 5 mg/ml BSA.

ing enzyme activity before and after fixation and permeabilization. PM (150  $\mu$ g) was fixed according to Method I and incubated in suspension for 18 h at 4°C in the presence or absence of 0.02% saponin (Treatment B, Table I, except that antibody was not added). Samples were removed for enzyme determinations and the remainder was centrifuged for 30 min at 100,000 g. The supernates were removed, the pellets were resuspended in PBS containing 0.25 M sucrose, and both fractions were assayed for LAP activity as previously described (8).

### Morphometric Analysis

The percent distribution of PA-Au in the embedded PM fraction was determined in three separate plasma membrane preparations. Random photographs of the membranes were taken at  $\times 16,000$  and the distribution of PA-Au was determined on micrographs at  $\times 40,000$ . The following categories were scored: (a) sinusoidal membrane; (b) bile front membrane; (c) lateral membrane; (d) filaments; (e) other; and (f) undetermined. The sinusoidal, lateral, and bile front membranes were identified by their morphological appearance as described by Hubbard et al. (11). About 20% of the membranes in this PM preparation came from sources other than plasmalemma, including rough and smooth microsomes and mitochondria (11). These non-PM membranes were classified as "other." Membrane profiles that were PM but could not be assigned to a particular domain (e.g., sinusoidal membrane not in obvious association with the lateral surface and bile front membrane whose identifying tight junctions or connection with an identifiable bile front were not apparent in that particular section) were classified as "undetermined" and constituted ~7% of all the membrane profiles that had gold particles associated with them. Where membrane morphology was well preserved (Method II, D), the density of PA-Au particles along a linear length of membrane was determined by the method of Weibel et al. (14) as previously described (11). In regions where the membranes were sectioned tangentially, the free edges of the bilayer were joined by a line that followed the contour of the interrupted membrane (see Fig. 4).

### Biochemical Studies Related to the Immunolabeling of Embedded Plasma Membranes

**DETERMINATION OF OPTIMUM ANTIBODY CONCENTRATIONS:** A solid-phase binding assay was used to standardize the amounts of protein A-reactive antibodies used in the agarose labeling methods described above. The procedure of D. P. Kiehart, D. A. Kaiser, and T. D. Pollard (manuscript submitted) was used with the following modifications: (a) 0.1  $\mu$ g of protein A

in 10 mM imidazole, pH 7.7, was dried down on a microtiter well (Dynatech Laboratories, Inc. Alexandria, VA); (b) antibody incubations were 2 h at room temperature; (c)  $^{125}$ I-goat anti-guinea pig IgG ( $5.0 \times 10^5$  cpm) was used to quantitate antibody binding to protein A; and (d) the amount of radioactivity bound to the microtiter well was corrected for the amount of  $^{125}$ I-goat anti-guinea pig bound to a well in the absence of added guinea pig antibodies (~20% of guinea pig value).

**ANALYTICAL PROCEDURES:** Enzyme assays and iodinations were carried out as described in reference 8.

## RESULTS

### Morphology of the Plasma Membrane Fraction

The ultrastructural appearance of prefixed PM embedded in agarose was very similar to that recently described by Hubbard et al. (see Figs. 1 and 2 in reference 11). The three plasmalemmal domains could be readily identified in PM not exposed to detergent (Fig. 1).

Our earlier immunofluorescence study (8) indicated that LAP was concentrated in the BC domain of the hepatocyte. At the ultrastructural level the bile canalicular domain in isolated PM was characterized by small vesicle profiles enclosed within an encircling membrane and was set off from the lateral surface by tight junctions. The close apposition of the membranes at the region of the tight junction and the meshwork of desmosomal filaments immediately beyond the junctions were hallmarks we used to identify this domain. When saponin was included in the incubation mixture, the membranes showed variable degrees of disruption. With the highest concentration of saponin (0.02%), the membrane morphology was altered and the overall organization of the membrane sheets was less apparent (see below). However, the three membrane domains could still be identified. With lower saponin concentration (0.005%) the membranes were less disrupted, however, they also showed a reduced permeability to colloidal gold (see below).

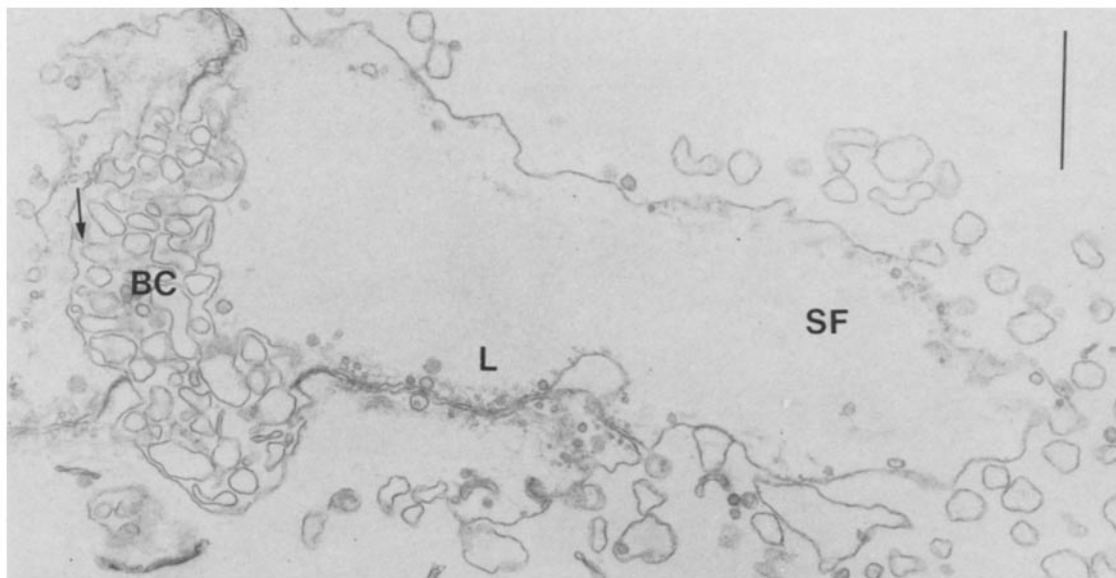


FIGURE 1 The distribution of PA-Au on embedded plasma membranes. Prefixed PM was embedded in agarose and incubated with anti-LAP then PA-Au, in the absence of detergent. The distinctive morphology of the three PM domains is evident. The bile canalicular domain (BC) consists of small vesicle profiles within an encircling membrane; this domain is set off from the lateral surface (L) by tight junctions. The lateral surface appears as parallel strips of membrane connecting the BC and sinusoidal (SF) domains. The sinusoidal domain is comprised of numerous loose irregular vesicle profiles and membrane continuous with the lateral surface. Very few gold particles are present in any of the domains, although one or two (arrow) can be seen. Bar, 1  $\mu$ m.  $\times 18,600$ .

## Effect of Fixation and Saponin Permeabilization on LAP Activity

Fixation of the membranes in 2% formaldehyde, 0.1% glutaraldehyde resulted in a 30% decrease in LAP activity, and overnight incubation at 4°C saponin resulted in a further 20% loss (Table II). However, saponin did not release significant amounts of enzyme activity from the plasma membrane sheets since 99% of the postincubation activity was present in the resuspended pellet after centrifugation at 100,000 *g*. We did not detect an increase in LAP activity with saponin treatment (i.e., latent activity), possibly owing to the small size (288 dalton) and hydrophobicity of the nitroanilide substrate used for these studies.

## Localization of LAP in Isolated Plasma Membranes

A number of different fixation and permeabilization conditions were tested (Table I) in an attempt to optimize the immunolabeling of the embedded plasma membrane sheets. The results obtained with three different fixation-permeabilization protocols are presented below.

**DISTRIBUTION OF COLLOIDAL GOLD PARTICLES ON UNPERMEABILIZED PLASMA MEMBRANES:** When plasma membranes fixed with 2% formaldehyde-0.1% glutaraldehyde but not permeabilized (Method A) were incubated with immune or nonimmune antibodies followed by PA-Au, small clusters of isolated gold particles were observed on the

TABLE II  
Saponin Solubilization of LAP Activity from Fixed Plasma Membranes

Sample	Saponin	Activity* μmol/h	Recovery* %
Unfixed membranes	—	2.78	100
Fixed membranes	—	1.92	69
Postincubation <sup>§</sup>	—	1.36	49
	+	1.32	47
100,000 <i>g</i> supernate	—	.01	0.3
	+	.02	0.7
100,000 <i>g</i> pellet	—	1.33	48
	+	1.34	49

\* These numbers represent the average of two experiments.

† The percent recovery given is relative to unfixed membranes.

§ Fixed membranes were incubated for 18 h at 4°C in the presence or absence of 0.02% saponin.

membrane sheets (Fig. 1). These particles were found on the cytoplasmic aspect of the membranes and were most often associated with filamentous material. Occasionally, labeled bile front membranes were observed in samples incubated with anti-LAP. These images accounted for ~3% of all bile fronts examined and most likely represent membranes disrupted during the isolation, fixation, or embedding process. The distribution of gold particles among the five categories is presented in Table III, where it is apparent that the sinusoidal and lateral membranes were labeled to similar extents with both immune and nonimmune antibodies. The filaments were also labeled to nearly the same extent in both immune and nonimmune samples (Table III, immune 30%, nonimmune 54.6%). The association of PA-Au with filaments represented nonspecific labeling, not labeling of LAP, for several reasons: (a) omission of BSA increased filament labeling, but variations in ionic strength (0–0.5 M NaCl) had no effect (data not shown); and (b) extraction of the membranes under conditions known to release cytoplasmic filaments (15) did not decrease the amount of LAP activity associated with the plasma membranes.

**DISTRIBUTION OF COLLOIDAL GOLD PARTICLES ON SAPONIN-PERMEABILIZED PLASMA MEMBRANES:** When either high or low concentrations of saponin were added to permeabilize the prefixed membranes (Treatments B and C, Table I), significant increases in the labeling of the bile front domain were observed with immune antibodies (Fig. 2 and Table III). Quantitation revealed that samples incubated with either 0.02% or 0.005% saponin had about 20 times more gold particles per bile front than did those incubated with nonimmune IgG (Table III). Morphologically, the three domains could still be recognized after saponin treatment, but the membranes were substantially disrupted by 0.02% saponin (Fig. 2, *a* and *c*). The microvilli within the bile canaliculi, as well as the outer encircling membrane, could not clearly be defined in these membranes. Nonetheless, in the immune sample (Fig. 2*a*), gold particles appeared uniformly distributed across sectioned bile fronts. The other membranes present in the embedded-permeabilized preparations incubated with immune antibody demonstrated the same level of labeling as that seen in the nonimmune control (Table III).

When agarose squares were incubated with a lower concentration of saponin (0.005%, labeling protocol C), the membrane domains retained much more of their characteristic morphology (Fig. 2, *b* and *d*) but were labeled to a lesser extent than when 0.02% saponin was used (124 vs. 300

TABLE III  
Distribution of Protein A-Gold on Plasma Membrane Sheets after Sequential Fixation and Saponin Permeabilization

Saponin*	Anti-body†	Distribution (%)						Undetermined	Total particles scored
		Sinusoidal	Bile	Lateral	Filament	Other			
None (A)	I	23.0 <sup>‡</sup> (10) <sup>‡</sup>	3.8 (10)	8.9 (14)	30.0 (16)	2.8 (6)	32.0 (6)	1,437	
	NI	20.6 (7)	13.0 (7)	6.3 (9)	54.6 (12)	0 (12)	5.6 (12)	621	
High (B) (0.02%)	I	9.6 (14)	62.0 (13)	8.0 (18)	12.0 (18)	2.0 (7)	6.5 (11)	6,252	
	NI	32.5 (8)	13.0 (8)	10.5 (9)	36.6 (13)	1.0 (4)	6.3 (5)	897	
Low (C) (0.005%)	I	12.0 (6)	48.0 (10)	5.4 (11)	32.0 (13)	2.4 (2)	0.2 (2)	2,623	
	NI	28.5 (8)	3.4 (8)	5.5 (11)	60.0 (11)	1.8 (4)	0.4 (1)	1,075	

\* See Table I for brief description of PM treatments A, B, and C.

† I, immune; affinity-purified anti-LAP; NI, nonimmune IgG.

‡ Percent of the total number of particles scored associated with the specified domain or structure. All gold particles associated with membranes were scored regardless of disposition on cytoplasmic or luminal side of the bilayer.

§ Number of profiles scored.

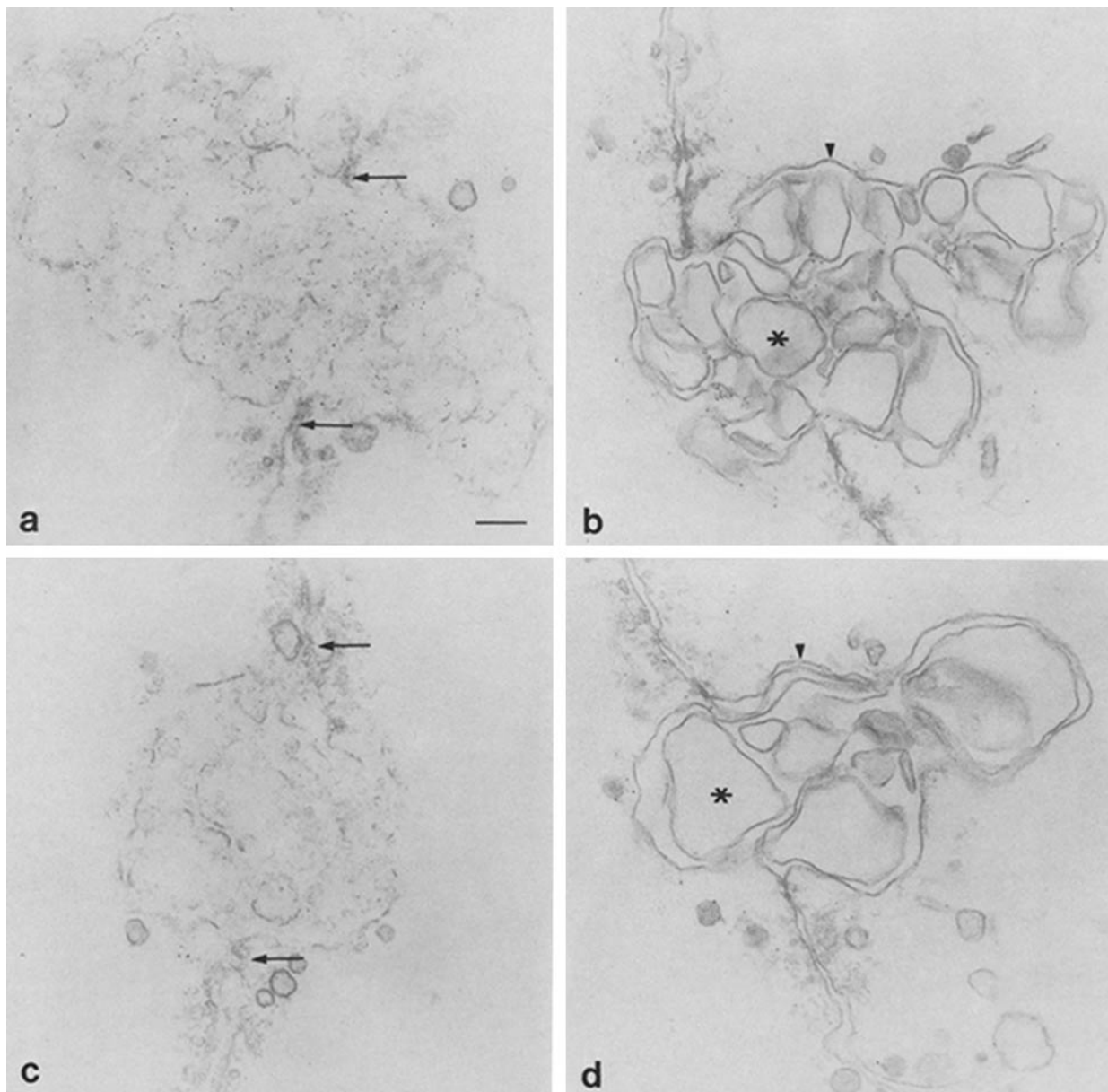


FIGURE 2 Distribution of PA-Au on saponin-permeabilized bile canalicular membranes. (a and b) Embedded PM incubated with affinity-purified anti-LAP in the presence of 0.02% saponin (a, and Table I, Treatment B) or after permeabilization with 0.005% saponin (b, and Table I, Treatment C). (a) High concentrations of saponin (0.02%) dramatically alter the morphology of the PM, but the BC domain can still be identified as a region bound on both sides by dense filamentous areas (arrows). Gold particles are numerous and are scattered uniformly throughout this domain. (b) The morphology of the BC is better preserved when lower concentrations of saponin (0.005%) are used for briefer periods (Table I). Microvilli (asterisk) and the outer encircling membrane (arrowhead) can be recognized. Gold particles are abundant in this domain but are less numerous and less uniformly distributed than in the BC from PM exposed to higher levels of saponin and are clustered. (c and d) PM incubated with nonimmune IgG in the presence of 0.02% saponin or after exposure to 0.005% saponin. Very few gold particles are seen in the BC domains after either treatment. However, gold is associated with filaments in all samples, immune and nonimmune. This is nonspecific labeling and does not represent LAP. Bar, 0.2  $\mu\text{m}$ .  $\times 35,000$ .

particles per bile front, respectively, calculated from data in Table III). In addition, gold particles associated with the BC domain after exposure to 0.005% saponin were not uniformly distributed, but were clustered over regions of membrane where the bilayer could not readily be resolved. This difference in the distribution of PA-Au at the two saponin concentrations was most likely due to differences in the extent of membrane permeabilization and thus the accessibility of the labeling reagents to the antigen. The restricted diffusion of a

particulate tracer and its clustered distribution over membrane disruptions in aldehyde-fixed, saponin-permeabilized cells have been observed by others (16). In preliminary studies we found that  $^{125}\text{I}$ -PA or  $^{125}\text{I}$ -PA-Au binding to agarose embedded PM increased when the saponin concentration was increased from 0.005% to 0.02% (data not shown), indicating that more antigen was detected at the higher detergent concentration. Thus, despite the variability in the labeling pattern, these initial localization studies indicated that LAP was highly

concentrated in bile front membrane. However, the membrane disruption observed at high saponin concentrations and the reduced labeling seen at lower concentrations prompted us to investigate other fixation-permeabilization methods to better visualize the distribution of LAP within the bile front domain.

**DISTRIBUTION OF COLLOIDAL GOLD PARTICLES ON SIMULTANEOUSLY FIXED AND PERMEABILIZED PLASMA MEMBRANES:** The simultaneous fixation-permeabilization conditions described by Zeitlin (17) for isolated rat hepatocytes resulted in significant antibody labeling of agarose-embedded membranes while retaining the morphology of the plasmalemmal domains to a greater extent than did sequential fixation and permeabilization. The labeling pattern we obtained with immune and nonimmune antibody preparations is presented in Fig. 3. The distribution of gold particles in samples incubated with anti-LAP antibodies was fairly uniform across sectioned bile fronts, and in general was associated with the luminal aspect of the membrane (Figs. 3a and 4a). The results of a distributional analysis are presented in Table IV. With anti-LAP antibodies, significant labeling of bile front membranes was obtained. 64% of all of the gold particles scored were present in this membrane domain. The

labeling of sinusoidal and lateral membranes was comparable to that observed in the samples incubated with nonimmune guinea pig IgG.

Since membrane morphology was better preserved using the simultaneous fixation-permeabilization protocol, we were able to determine the labeling density in each of the three PM domains (i.e., number of gold particles per unit length of membrane). Fig. 4a is an example of a micrograph used for these measurements, the results of which are presented in Table IV. After saponin permeabilization and in the presence of immune antibodies, the bile canalicular domain was the only category that was significantly labeled, and the density of gold associated with the BC domain was 5–10 times higher than that associated with either the lateral or sinusoidal domains. From these studies we conclude that LAP is highly enriched in the bile front domain in isolated plasma membranes and that the antigen seems to be fairly uniformly distributed along the canalicular membrane and not clustered into discernible foci. The lateral and sinusoidal membranes appear not to contain significant quantities of LAP.

## DISCUSSION

In the present study we have localized LAP at the ultrastruc-

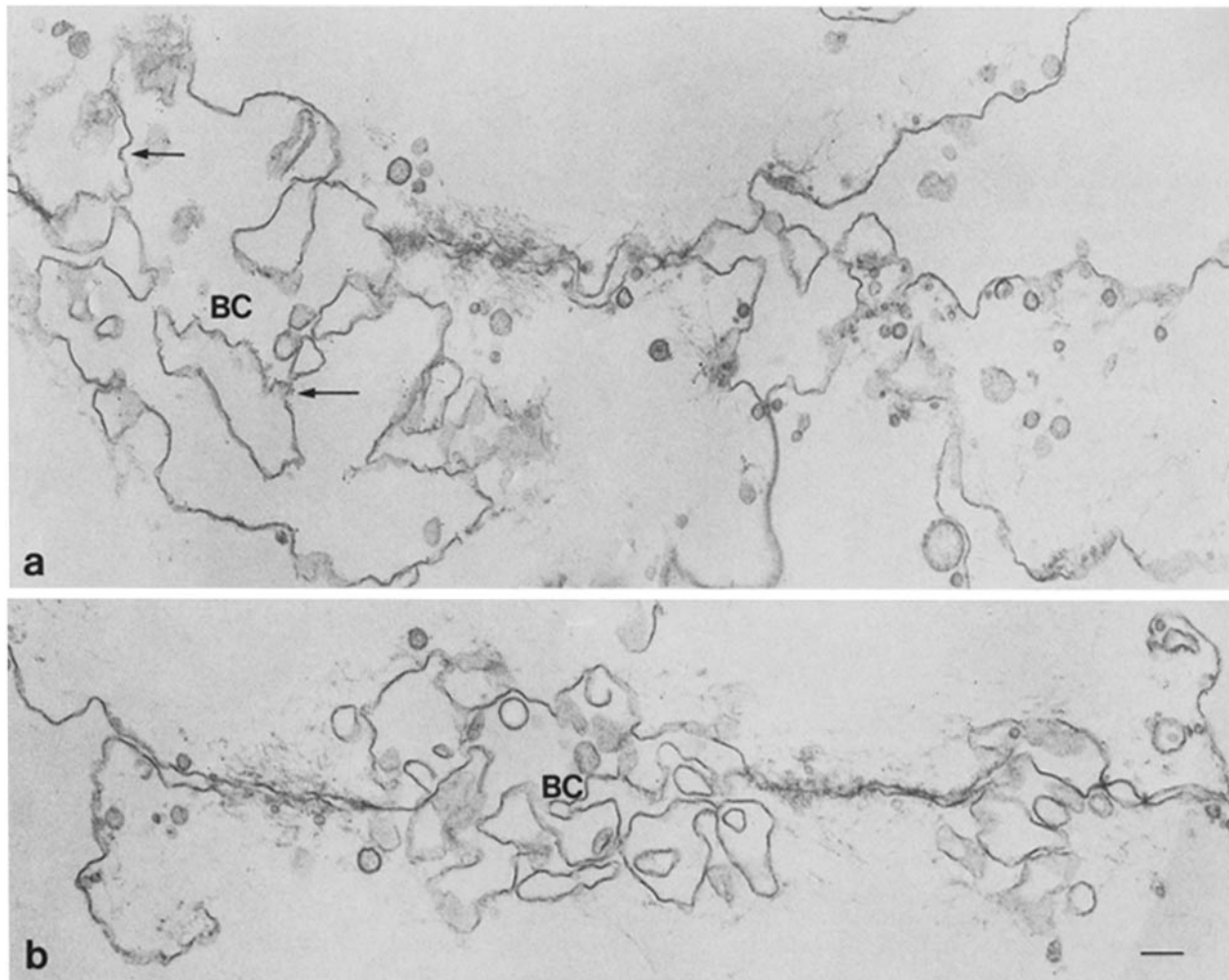


FIGURE 3 Distribution of PA-Au on simultaneously fixed and permeabilized plasma membranes. Embedded plasma membranes were incubated with affinity-purified anti-LAP (a) or nonimmune IgG (b) followed by PA-Au (Table I, Treatment D). Clusters of gold particles (arrows) are distributed along BC membrane in the presence of specific antibody (a) but are absent in the nonimmune control (b). The filaments, as well as the lateral and sinusoidal membranes, are labeled to the same extent in both samples. Bar, 0.2  $\mu\text{m}$ .  $\times 27,000$ .

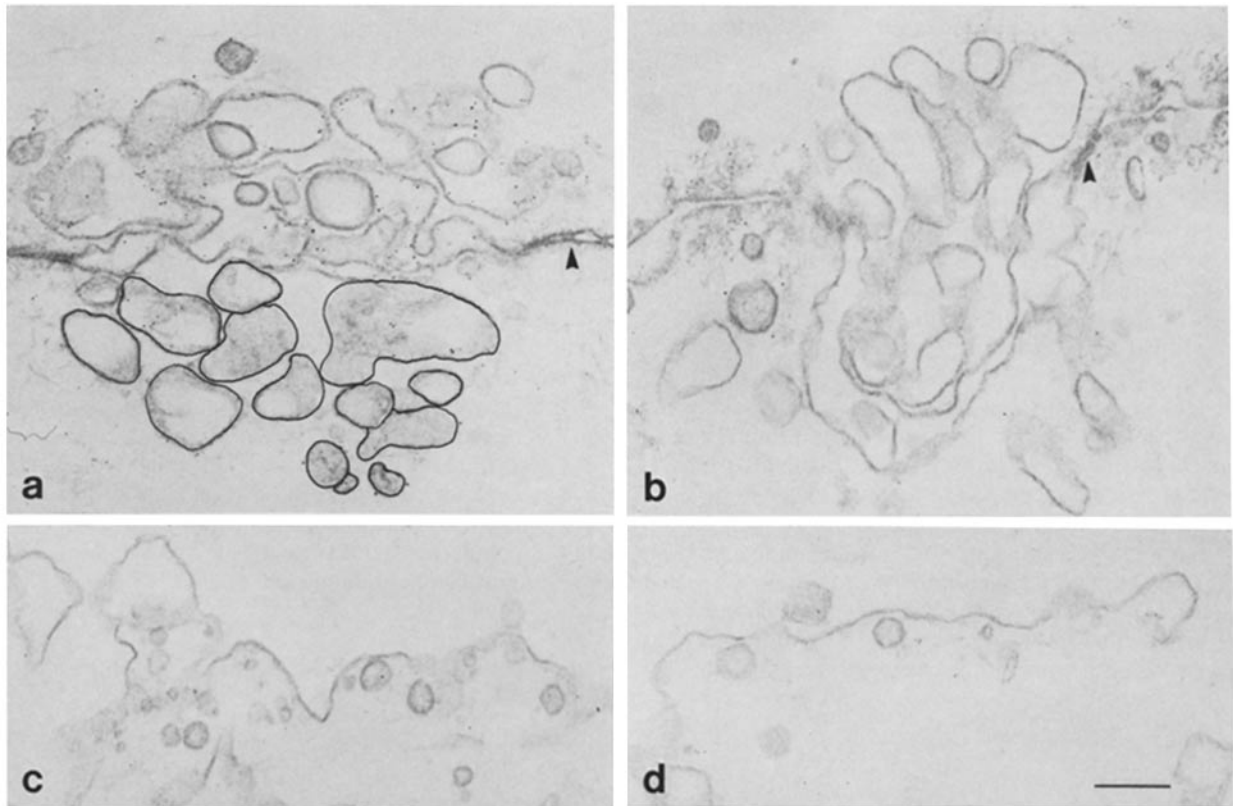


FIGURE 4 Distribution of PA-Au along the bile front and sinusoidal membranes of simultaneously fixed and permeabilized PM. Higher magnifications of the canalicular and sinusoidal domains from PM sheets fixed and permeabilized under the same conditions as those used in Fig. 3. (a) Immune (b) Nonimmune. Tight junctions (arrowheads) are evident. Half of the bile front in a has been outlined to demonstrate how the micrographs were prepared for morphometry. (c and d) Distribution of PA-Au along sinusoidal membranes. (c) Immune. (d) Nonimmune. Gold particles are associated with the luminal and cytoplasmic aspects of the PM as well as with filaments. Bar, 0.2  $\mu\text{m}$ .  $\times 50,000$ .

TABLE IV  
Distribution of Protein A-Gold on Plasma Membrane Sheets after Simultaneous Fixation and Permeabilization

Treatment*	Antibody	Distribution	Sinusoid	Bile	Lateral	Filaments	Other	Undetermined	Total particles scored
A (No detergent)	I	% <sup>†</sup>	15.3 (7)	17.4 (7)	6.4 (7)	27.4 (9)	13.4 (7)	20.0 (7)	798
		P/L <sup>‡</sup>	0.84	1.17	0.85				
	NI	%	14.6 (5)	17.0 (6)	6.3 (6)	47.0 (9)	12.2 (9)	2.8 (3)	662
D (Simultaneous fix-permeabilize)	I	%	6.8 (6)	64.8 (8)	1.7 (1.0)	16.0 (14)	6.0 (13)	4.8 (6)	2,177
		P/L	0.82	6.5	0.56				
	NI	%	7.4 (7)	11.2 (7)	5.1 (10)	67.7 (11)	6.6 (10)	2.0 (5)	848
		P/L	0.88	0.47	0.51				

I, immune; NI, nonimmune.

\* These data were obtained from two experiments. See Table I for a brief description of the treatments.

<sup>†</sup> The percent of the total number of particles scored associated with the specified domain of structure. All gold particles found within 18 nm were scored and no distinction was made between the cytoplasmic and luminal aspect of the bilayer. The number in the parentheses represents the number of profiles scored.

<sup>‡</sup> Morphometric analysis was performed as described in Materials and Methods on micrographs taken of the three domains of the embedded PM sheets. Gold particles were scored on the same micrographs and assigned to one of the categories listed above. Particles per linear length (P/L) = the number of particles per domain divided by the number of intersections per domain. The mean ( $\bar{x}$ ) is given. A Student's *t* test was carried out on these values. The results of this analysis showed that only the BC membrane of saponin permeabilized samples incubated with anti-LAP antibodies was specifically labeled.

tural level to the bile front domain of isolated rat liver plasma membrane sheets. This result extends our earlier report in which LAP was localized in situ by immunofluorescence to the same plasmalemmal domain.

#### Detection of Antigen in Agarose-embedded Fractions

The advantages of embedding subcellular fractions in agarose for immunolabeling have been thoroughly discussed by

deCamilli et al. (12). By embedding the plasma membrane fraction in agarose, we avoided the repetitive sedimentations and resuspensions that disrupt the domain morphology of the plasmalemmal sheets, and we also achieved better spatial separation of the membrane sheets. This second feature facilitated morphometric and quantitative analyses, since the total sample could be visualized and the three domains could be more readily identified than in membrane pellets.

Specific immunostaining of LAP in isolated plasma mem-



branes was only obtained after the membranes were permeabilized by detergent. Saponin was chosen as a permeabilizing agent for our studies, because others had previously demonstrated the entry of ferritin into aldehyde-fixed cells (16, 18) and cellular organelles (11, 12) after exposure to this detergent. In addition, membrane morphology appears to be better preserved after treatment with saponin as compared with Triton X-100 (19). We did not examine the ability of other detergents (e.g., Triton X-100 and Brij) to permeabilize the embedded membranes, so we do not know to what extent these reagents would allow specific labeling while maintaining domain morphology.

### Localization of LAP in Isolated Plasma Membranes

The distribution of LAP within the BC domain, as detected by PA-Au, varied according to the fixation-permeabilization protocol used. At a high saponin concentration (0.02%), the membranes were substantially disrupted and the gold particles appeared uniformly distributed across a sectioned bile front. At a low saponin concentration (0.005%), the membranes were largely intact but displayed a lower extent of labeling and a less random distribution of particles. The PA-Au in the latter samples was clustered over membrane regions where the bilayer could not be defined. Ohtsuki et al. (16) observed a similar clustering of ferritin particles over disruptions in aldehyde-fixed, saponin-permeabilized erythrocyte plasma membranes. They postulated that cross-linked cytoplasmic proteins restricted the diffusion of tracer into the cell. Thus, the patches of gold particles seen on BC membrane incubated with 0.005% saponin are probably the result of the poor penetration of the PA-Au and/or the IgG, owing to limited disruption of the bilayer. From the PA-Au distribution obtained with the other two labeling protocols, we conclude that LAP is present throughout the BC.

Based on the specific LAP activity in the isolated PM fraction, we estimate that there are  $2.5 \times 10^5$  molecules of LAP per cell. Making several assumptions about section thickness (100 nm), the number of bile fronts per cell (3), and the diameter of a bile front profile (2  $\mu\text{m}$ ), we should have detected about 400 gold particles per sectioned bile front. The average number of particles we scored in one bile front was 320 (with 0.02% saponin;  $320 \pm 123$ ,  $n = 12$ ). Since there may be fewer than three such fronts per cell, and antigenicity may not be decreased by fixation and incubation to the same extent as LAP activity, the number of LAP molecules detected by our protocol and the expected number are in reasonable agreement. With the simultaneous fixation-permeabilization method, we visualized only ~15% of the antigen. Since the maximum labeling was obtained with the highest degree of membrane disruption, we do not know at present if small clusters of antigen exist along the BC membrane.

Emmelot et al. (20) proposed that LAP was associated with 5–6-nm globular knobs in isolated rat liver plasma membranes, and that these knobs were concentrated in the BC domain. Their evidence was based on (a) the association of LAP with globular units isolated from intestinal brush border membrane (21); (b) the detection by negative staining of knobs restricted to only a few regions of the hepatocyte plasmalemma; (c) the parallel release of these structures and LAP activity from the hepatocyte membrane, without the release of 5'-nucleotidase, alkaline phosphatase, or alkaline phospho-

diesterase; and (d) the histochemical localization of LAP to the liver bile canalicular domain (22, 23). Others have also reported the ability of proteases to remove globular knobs and LAP activity from isolated liver PM sheets (24, 25). Unfortunately, none of these investigators determined whether LAP was the only protein associated with the globular units or whether all knobs carried this enzyme. In addition, negative staining is not sensitive enough to assess the distribution of small protuberances along the plasmalemma. Thus, the evidence for an association of liver PM LAP with globular units remains indirect. Although we have not localized LAP to globular knobs in this study, we have directly shown that PM-LAP is concentrated in BC membrane.

### Specific Immunostaining of the Sinusoidal and Lateral Membranes Was Not Detected

Several reports on the biosynthesis of sucrase-isomaltase (5) and the recycling of LAP (26) have suggested that these integral membrane proteins first appear in the basolateral membrane of cells before their concentration in the apical domain. We did not detect specific LAP on the lateral or sinusoidal surfaces in isolated PM. However three observations prevent us from concluding that LAP is absent from these domains. First, we did find gold associated with lateral and sinusoidal membrane in both immune and nonimmune samples; however, the absolute extent of labeling (PA-Au per front) was similar in both samples. Nevertheless, such background labeling prevents an accurate evaluation of the number of LAP molecules present in these two membrane domains. Second, the dense network of cytoplasmic filaments may restrict the penetration of antibody and gold tracer through the lateral membrane. The ability of aldehyde-cross-linked cytoplasmic proteins to act as diffusion barriers to macromolecules has been demonstrated (12, 16). However, occasional particles were found associated with the outer aspect of the lateral membrane immediately beyond the tight junctions suggesting that some access to this domain was obtained. Lastly, the plasma membranes used represent only ~20% of the total plasmalemma and the ratios of the three membrane domains in this fraction are different from those reported for cells in situ (11, 27). Thus it is not known to what extent, if any, we are selecting for a population of bile fronts that is enriched in LAP. Immunofluorescent staining of cryostat sections of liver suggested that all identifiable BC contained LAP, however the entire liver was not systematically examined to determine if all regions of a lobe as well as each lobe of the liver contained the same pattern of labeling and number of reactive BC. Notwithstanding these considerations, the lack of specific labeling of the lateral and sinusoidal membrane in the PM preparation used for this study and the concentration of LAP in BF membrane suggest that LAP is a useful probe for the affinity isolation of BF membranes. In the accompanying paper (28), we show that anti-LAP-coated *Staphylococcus aureus* cells can be used to isolate bile front membrane from a sonicated PM preparation.

We would like to thank Ms. A. Daniel for the preparation of the manuscript, Mr. T. Urquhart for his photographic work, and Dr. P. deCamilli for giving us the full details of his agarose embedment method before its publication.

This project was supported by a National Institutes of Health grant to Ann Hubbard (GM 29185).



## REFERENCES

1. Louvard, D., S. Maroux, J. Baratti, P. Desnuelle, and S. Mutaftschiev. 1973. On the preparation and some properties of closed membrane vesicles from hog duodenal and jejunal brush border. *Biochim. Biophys. Acta.* 291:747-763.
2. George, S. G., and A. J. Kenny. 1973. Studies on the enzymology of purified preparations of brush border from rabbit kidney. *Biochem. J.* 134:43-57.
3. Vannier, C., D. Louvard, S. Maroux, and P. Desnuelle. 1976. Structural and topological homology between porcine intestinal and renal brush border aminopeptidase. *Biochim. Biophys. Acta.* 455:185-199.
4. Ziomek, C. A., S. Schulman, and M. Edidin. 1980. Redistribution of membrane proteins in isolated mouse intestinal epithelial cells. *J. Cell Biol.* 86:849-857.
5. Hauri, H.-P., A. Quaroni, and K. J. Isselbacher. 1979. Biogenesis of intestinal plasma membrane: posttranslational route and cleavage of sucrose-isomaltase. *Proc. Natl. Acad. Sci. USA.* 76:5183-5186.
6. Fujita, M., H. Ohta, K. Kawai, H. Matsui, and M. Nakao. 1972. Differential isolation of microvillous and basolateral plasma membrane from intestinal mucosa. Mutually exclusive distribution of digestive enzymes and ouabain-sensitive ATPase. *Biochim. Biophys. Acta.* 274:336-347.
7. Scaleria, V., Y.-K. Huang, B. Hildmann, and H. Murer. 1981. A simple isolation method for basolateral plasma membrane from rat kidney cortex. *Membr. Biochem.* 4:49-61.
8. 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.
9. Roman, L. M., and A. L. Hubbard. 1983. Isolation of rat liver canalicular membrane by immunoadsorption. *J. Cell Biol.* 97 (2, Pt. 2): 304a. (Abstr.)
10. Rash, J. E., T. J. Johnson, C. S. Hudson, F. D. Giddings, W. F. Graham, and M. E. Eldeffrawi. 1981. Labelled-replica techniques: post-shadow labelling of intramembrane particles in freeze-fracture replicas. *J. Microsc. (Oxf).* 128:121-138.
11. Hubbard, A. L., D. A. Wall, and A. Ma. 1983. Isolation of rat hepatocyte plasma membranes. I. Presence of the three major domains. *J. Cell Biol.* 96:217-229.
12. deCamilli, P., S. M. Harris, W. B. Huttner, and P. Greengard. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.* 96:1355-1373.
13. Hubbard, A. L., G. Wilson, G. Ashwell, and H. Stukenbrok. 1979. An electron microscope autoradiographic study of the carbohydrate recognition system in rat liver. I. Distribution of <sup>125</sup>I-ligands among the liver cell types. *J. Cell Biol.* 83:47-64.
14. Weibel, E. R., W. Staubli, H. R. Gnagnni, and F. A. Hess. 1969. Correlated morphometric and biochemical studies on the liver cell. I. Morphometric model, stereologic methods, and normal data for rat liver. *J. Cell Biol.* 42:68-91.
15. Hubbard, A. L., and A. Ma. 1983. Isolation of rat hepatocyte plasma membranes. II. Identification of membrane-associated cytoskeletal proteins. *J. Cell Biol.* 96:230-239.
16. Ohtsuki, I., M. Manzi, G. E. Palade, and J. D. Jamieson. 1978. Entry of macromolecular tracers into cells fixed with low concentrations of aldehydes. *Biol. Cell.* 31:119-126.
17. Zeitlin, P. L. 1983. Plasma membrane domain-specific molecules in rat hepatocytes. Ph. D. thesis. Yale University, New Haven, CT.
18. Seeman, P. 1967. Transient holes in the erythrocyte membrane during hypotonic hemolysis and stable holes in the membrane after lysis by saponin and lyssolecithin. *J. Cell Biol.* 32:55-70.
19. Wehland, J., M. C. Willingham, M. G. Gallo, and I. Pastan. 1981. The morphologic pathway of exocytosis of the vesicular stomatitis virus G protein in cultured fibroblasts. *Cell.* 28:831-841.
20. Emmelot, P., A. Visser, and E. L. Benedetti. 1968. Studies on plasma membranes. VII. A leucyl- $\beta$ -naphthylamidase-containing repeating unit on the surface of isolated liver and hepatoma plasma membranes. *Biochim. Biophys. Acta.* 150:364-375.
21. Overton, J., A. Eichholz, and R. K. Crane. 1965. Studies on the organization of the brush border in intestinal epithelium cells. II. Fine structure of fractions of Tris-disrupted hamster brush borders. *J. Cell Biol.* 26:693-706.
22. Nachlas, M. M., B. Monis, D. Rosenblatt, and A. M. Seligman. 1960. Improvement in the histochemical localization of leucine aminopeptidase with a new substrate, L-leucyl-4-methoxy-2-naphthylamide. *J. Biophys. Biochem. Cytol.* 8:413-422.
23. Monis, B. 1965. Isozymes of arylamidase (aminopeptidase): source of blood aminoacyl naphthylamidase in the rat. *Nature (Lond.)* 208:587-588.
24. Oda, M., I. M. Yousef, K. Funatsu, V. D. A. Edwards, and M. J. Phillips. 1982. Electron cytochemical and biochemical analysis of canalculus-enriched liver cell membranes. *J. Ultrastruct. Res.* 78:26-39.
25. Oda, M., and M. J. Phillips. 1975. Electron microscopic cytochemical characterization of bile canaliculi and bile ducts *in vitro*. *Virchows Arch. B Cell Pathol.* 18:109-118.
26. Louvard, D. 1980. Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA.* 77:4132-4136.
27. Weibel, E. R. 1973. Stereological approach to the study of cell surface morphometry. European Congress on Electron Microscopy, 6th, Jerusalem. 6-9.
28. Roman, L. M., and A. L. Hubbard. 1983. A domain-specific marker for the hepatocyte plasma membrane. III. Isolation of bile canalicular membrane by immunoadsorption. *J. Cell Biol.* 98:1497-1504.