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ISOLATION OF A RAT LIVER PLASMA MEMBRANE FRACTION OF PROBABLE CANALICULAR ORIGIN

PREPARATIVE TECHNIQUE, ENZYMATIC PROFILE, COMPOSITION, AND SOLUTE TRANSPORT

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A technique currently used for isolation of brush border membranes from renal and intestinal epithelium that involves vigorous tissue homogenization and sedimentation of non-luminal membranes in the presence of Mg^{2+} has been adapted to rat liver. Liver plasma membranes so prepared consisted almost exclusively of vesicles by electron microscopy, showed some contamination with endoplasmic reticulum and minimal contamination with mitochondria or Golgi by marker enzymes, were highly enriched in alkaline phosphatase, Mg^{2+}-ATPase, and 5′-nucleotidase activity compared with homogenate, and showed little enrichment in (Na^+, K^+)-ATPase. Comparison of this enzymatic profile with cytochemical studies localizing (Na^+, K^+)-ATPase and alkaline phosphatase to the sinusoidal/lateral and canalicular membranes, respectively, suggested that these membranes were predominantly of canalicular origin. They had a lower ($Na^+ + K^+$)-ATPase specific activity, lower lipid content, and higher cholesterol to phospholipid molar ratio than a conventional plasma membrane preparation believed to be enriched in canaliculi. Moreover, it was possible to measure movement of D-[^{3}H]glucose into an osmotically sensitive space bounded by these membrane vesicles.

Introduction

The plasma membrane of secretory and absorptive epithelial cells consists of at least two distinct portions. One portion (luminal) faces the lumen into which fluid is secreted or from which fluid is absorbed. The other portion (antiluminal) is in contact with the interstitial or vascular compartment. These surfaces are separated by junctional complexes which perform a variety of functions including the joining of adjacent epithelial cells. Recently reported methods for the selective isolation of luminal (brush border) [1–9] and antiluminal (basolateral) membranes from renal and intestinal epithelial cells have allowed direct study of the transport mechanisms operative at opposite poles of these cells and have greatly enhanced current understanding of absorptive epithelia [10,11].

The hepatocyte plasma membrane also consists of a luminal portion (canalicular membrane) separated from an antiluminal portion (sinusoidal and lateral membranes) by a junctional complex [12]. Most techniques currently used for preparation of liver plasma membranes involve gentle tissue homogenization followed by sucrose gradient centrifugation [13–30]. These techniques generally yield a morphologically heterogeneous preparation consisting of vesicles and membrane sheets joined by tight junctions [16,19,21–28] which very likely contain membranes from all portions of the hepatocyte surface [28]. Kremmer et al. [31] have reported separate

Abbreviations: Mg^{2+}-ATPase, Mg^{2+}-activated adenosinetriphosphatase; ($Na^+ + K^+$)-ATPase, ($Na^+ + K^+$)-activated adenosinetriphosphatase; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

isolation of the sinusoidal, lateral and canalicular portion of the rat hepatocyte plasma membrane using a combination of differential, rate-zonal and density gradient centrifugation. These three subfractions showed differences in enzymatic activity but were similar in their overall content of cholesterol and phospholipids [32]. Two other groups of investigators have also reported the differential isolation of sinusoidal/lateral and canalicular membranes [23,24,32]. In both instances, however, the 'canalicular' fraction was found to be highly enriched in $(Na^+ + K^+)$-activated adenosinetriphosphatase ($Na^+ + K^+$)-ATPase) [24,32].

The present studies describe the isolation and characterization of a new liver plasma membrane fraction. On the premise that the luminal (canalicular) membrane of the hepatocyte may be analogous to the luminal (brush border) membrane of absorptive epithelia, we have applied to rat liver a simple technique currently used for isolation of renal and intestinal brush border membranes [1,7]. This technique differs from conventional preparative techniques [13,32] in that it involves vigorous tissue homogenization and sedimentation of non-luminal membranes in the presence of Ca^{2+} or Mg^{2+}. Our findings confirm that sinusoidal/lateral as well as mitochondrial and microsomal membranes precipitate during low speed centrifugation in the presence of Mg^{2+}. The membrane fraction prepared using this technique consisted almost exclusively of vesicles and showed enzymatic characteristics suggestive of an origin from canalicular microvilli and similar to those of renal and intestinal brush border membranes. This preparation differed significantly in both enzymatic profile and lipid composition from a 'canaliculi-enriched' membrane fraction perpared by us using a conventional technique [14,19]. Moreover, it was possible to demonstrate movement of radiolabeled D-glucose into an osmotically sensitive intravesicular space bounded by these membranes. The present findings suggest that canalicular and sinusoidal/lateral hepatocyte plasma membranes differ in lipid composition as well as enzymatic profile. Moreover, study of solute movement into vesicles composed of plasma membranes from different portions of the hepatocyte surface is possible and likely to provide valuable insight into hepatic transport.

Materials and Methods

Animals. Non-fasted male Sprague-Dawley rats (244–330 g) fed standard chow (Berkeley Standard Diet, Feedstuff Processing Company, San Francisco, CA) were used in all experiments. Animals were kept in wire bottom cages and maintained in a constant temperature environment (25°C) with alternating 12 h light and dark cycles.

Chemicals and radioisotopes. Unlabeled D-glucose (Sigma, St. Louis, MO), D-raffinose (Sigma), phlorizin (Sigma), phloretin (Sigma), mannitol (Sigma), sodium azide (Sigma), deoxycholate (Sigma), and ^{3}H-labeled and ^{14}C-labeled D-glucose (New England Nuclear, Boston, MA) were used as supplied by the manufactures.

Preparation of liver plasma membranes. Conventional liver plasma membranes 'enriched in canaliculi' were prepared by discontinuous sucrose density gradient centrifugation using a modification of the methods of Song et al. [14] and Boyer and Reno [19] as previously described [28,31]. These liver plasma membranes are subsequently referred to as preparation B.

Liver plasma membranes (preparation A) were prepared using an adaptation of the technique originally described by Schmitz et al. [7] and subsequently modified by Booth and Kenney [1] and Kessler et al. [9]. All steps were carried out in a cold room or on ice, and a Sorvall superspeed RC2-B refrigerated centrifuge and Sorvall SS 34 rotor were used for all centrifugations. Rats were sacrificed by decapitation and the liver was excised and perfused with ice-cold buffer A (50 mM mannitol, 2 mM Tris-HCl, pH 7.0 at 25°C) until all lobes were visibly blanched. Up to 20 g of perfused liver was added to 20 times the volume of buffer A and homogenized 4 min using a Sorvall Omnimix at full speed. The Omnimix bucket was kept in ice during homogenization to prevent heating. A 2-ml aliquot of homogenate was removed and stored at 4°C for later analysis, and the remaining homogenate was poured into a beaker containing sufficient $MgSO_4$ to yield a final homogenate concentration of 10 mM. (In preliminary experiments, $CaCl_2$ at the same concentration was used instead of $MgSO_4$ and similar results were obtained.) After stirring for 20 min, the homogenate-$MgSO_4$ mixture was poured into several tubes and centri-

fuged 15 min at 8 000 rev./min (7 700 × *g*). The pellets (P_1) were discarded and the supernatants (S_1) were carefully decanted into clean tubes and centrifuged at 13 000 rev./min (20 200 × *g*) for 30 min. The supernatants (S_2) following this centrifugation step were aspirated and discarded. The combined pellets (P_2) were mixed with a volume of buffer B (100 mM mannitol, 1 mM Tris, 1 mM Hepes, pH 7.5) equal to 10 times the original perfused liver weight and homogenized with six slow up-and-down strokes in a Potter-Elvehjem homogenizer. This homogenate was again poured into tubes and centrifuged 15 min at 8 000 rev./min (7 700 × *g*). The pellets (P_3) were discarded and the supernatants (S_3) were poured into clean tubes and centrifuged 25 min at 13 000 rev./min (20,000 × *g*). The supernatants (S_4) were discarded and the combined pellets (P_4) were transferred to conical 1.5 ml micro test tubes (BioRad, Richmond, CA) and resuspended in a small volume of buffer B. These containers were centrifuged 5 min at 4 000 rev./min (1 930 × *g*) to remove bacteria, and the supernatant (S_5) was transferred to clean conical 1.5 ml containers and centrifuged at 16 000 rev./min (30 900 × *g*) for 15 min to pellet the final preparation (P_6). This pellet (P_6) generally consisted of two reasonably distinct layers: a top layer which was yellowish-brown in color and an opalescent bottom layer. The top layer, which contained 80% of the total protein present in P_6 and 90 to 98% of total enzyme activity, was separated from the bottom layer (possibly representing glycogen) using a Pasteur pipette and was resuspended in a sufficient volume of buffer B (1–2 ml) to yield a protein concentration of 2–7 mg/ml. Preparation of a single batch of membranes by this technique required approx. 5 h. All membranes were kept at 4°C after preparation. Transport studies were completed within 6 h of preparation and measurements of enzyme activity and fluorescence polarization were performed the following day. Lipid determinations were performed on membranes frozen at –20°C for up to several weeks.

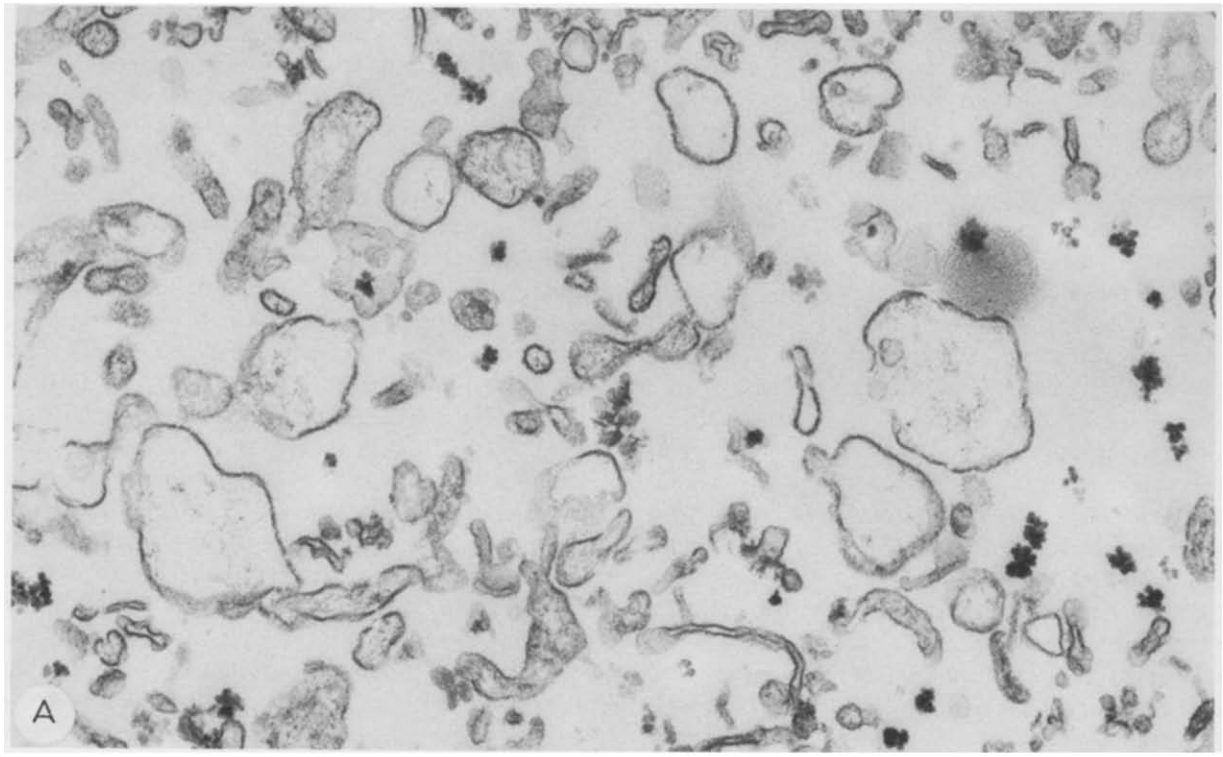
Enzyme assays. Mg^{2+}-activated adenosinetriphosphatase (Mg^{2+}-ATPase) and ($Na^+ + K^+$)-ATPase were measured using a recording spectrophotometric assay [33]. In selected studies, deoxycholate (1 or 4 mM) was added to the incubation mixture. Using a conventional assay [33], ATPase activity also was measured in the presence of Mg^{2+} and after addition of Na^+ as described by Proverbio et al. [34]. Alkaline phosphatase was measured by a modification of the method of Pekarthy et al. [35] in cuvettes containing 1.0 ml of reaction mixture, consisting of 46.7 μmol glycine (pH 10.5), 2.3 μmol of $MgSO_4$ and $ZnSO_4$, 5.6 μmol of *p*-nitrophenylphosphate, and 30–60 μg of membrane protein. The formation of *p*-nitrophenol at 37°C as measured on a recording spectrophotometer at 410 nm was linear to 15 min and proportional to the amount of membrane protein added. 5′-Nucleotidase was determined as previously described [26] except that the reaction was initiated by the addition of membranes and terminated at 10 min. Succinate-cytochrome *c* reductase, a mitochondrial enzyme, and NADPH-cytochrome *c* reductase, as microsomal enzyme, were measured as described previously [26]. UDPgalactose-*N*-acetylglucosamine galactosyltransferase activity was measured according to the method of Fleischer and Fleischer [36].

Lipid fluidity. Lipid fluidity was determined by fluorescence polarization using an Elscint MVIa microviscosimeter and the fluorescent hydrocarbon probe, 1,6-diphenyl-1,3,5-hexatriene (Aldrich Chemical Co., Inc., Milwaukee, WI) as previously described [28].

Lipid analysis. Membrane lipids were extracted according to the method of Folch et al. [37]. Unesterified cholesterol was quantified as outlined previously [28], and phospholipid was measured by the method of Bartlett [38].

Electron microscopy. Transmission electron microscopy was performed by Dr. Albert Jones of the Department of Medicine and Anatomy and the Liver Center, University of California, San Francisco, CA. Membrane preparations were fixed overnight at 4°C in 4% glutaraldehyde, concentrated by millipore filtration, postfixed in 2% osmium tetroxide buffered with 0.1 M cacodylate, pH 7.4, dehydrated in ethanol, and embedded in Epon 812. Thin sections were stained with uranyl acetate lead citrate and examined with a Phillips EM-300 electron microscope.

Transport studies. Uptake of radiolabeled D-glucose into membrane vesicles was measured essentially as described by Hopfer et al. [8], Evers et al. [39], and Berner and Kinne [2] for intestinal and renal microvillus membrane vesicles. Briefly, 110 μl of a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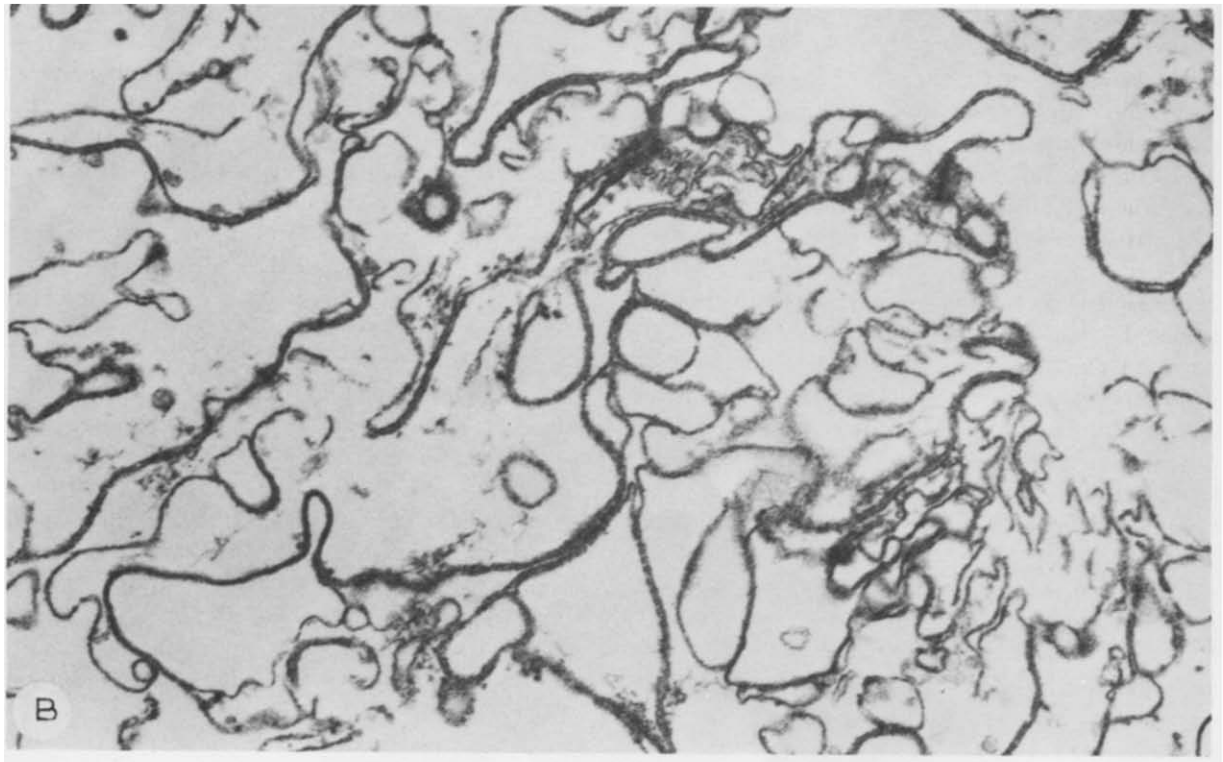
B

highly concentrated (5–20 mg protein/ml) membrane preparation in buffer B and 20 μl of 0.75 M NaCl in buffer B were placed in a 1.5 ml conical micro test tube (BioRad). At zero time, after the mixture had come to room temperature, 20 μl of a solution containing 3 μCi of D-[^{3}H]glucose and 7.5 or 3.75 mM unlabeled D-glucose was added and the container immediately vortexed. The final 150 μl incubation mixture thus contained 100 mM NaCl, 87 mM mannitol, 0.87 mM Tris-Hepes, 3 μCi D-[^{3}H]glucose and 1 or 50 mM unlabeled D-glucose. In studies intended to assess the effect of an Na^+ gradient on D-glucose uptake, 0.75 M NaCl was added to the 20 μl D-[^{3}H]-glucose containing solution added at the beginning of the uptake study rather than the 20 μl of buffer B added to the preincubation mixture. Some incubations also contained 100 μM phlorizin or 100 μM phloretin prepared by substituting 1.5 μl of 10 mM phlorizin or phloretin in 95%(v/v) ethanol for 1.5 μl of membrane preparation. No incubation contained more than 1%(v/v) ethanol and all studies were performed at room temperature.

Seven 20-μl aliquots were removed from this incubation mixture at various time intervals after addition of isotope (approx. 10, 45, 80 and 120 s, with triplicate samples between 15 and 16 min). These aliquots were transferred immediately to a test tube containing 1 ml of ice-cold 'stop' solution having the same composition as the incubation mixture but with 0.1 μCi/ml of D-[^{14}C]glucose instead of D-[^{3}H]glucose. The test tube was vortexed and the stop solution was transferred by Pasteur pipette to a 25 mm diameter, 0.45 μm pore size filter (Millipore type HA or Gelman Metrical types GN-6) on a filter holder mounted on an Erlenmeyer flask connected to a vacuum pump (VWR Scientific portable combination pressure/vacuum pump). Care was taken to ensure that all of the stop solution passed through the center of the filter without loss at the edge. When the stop solution had been completely filtered, the membrane vesicles and filter were washed with 5 ml of ice-cold solution (identical to the stop solution, but without D-[^{14}C]glucose) squirted onto the filter from a hand-held syringe. Transfer of each aliquot to the stop solution and suction filtration and washing of vesicles was completed in 20 s or less. The filters were dissolved in a counting vial containing 2 ml of ethylene glycol monomethyl ether to which 10 ml of scintillant (2 : 1 mixture of scintillation grade toluene and Triton X-100 containing 5 g/l of Preblend 2a70 (Research Products Int., Elk Grove Village, IL) was then added. Double isotope counting was performed in a Beckman LS 250 or LS 7 000 liquid scintillation counter with external standardization for quench correction. Approximately 20 μl of residual stop solution remaining in the test tube after transfer was also counted and the ratio of ^{14}C to ^{3}H was taken to be the ratio of ^{14}C- to ^{3}H-labeled D-glucose adherent to cells or present in extravesicular fluid, at the time of filtration. Dividing the ^{14}C dpm present in the counting vial by the ^{14}C/^{3}H dpm ratio present in the stop solution yielded the amount of D-[^{3}H]glucose which was adherent to rather than inside the membrane vesicles. This figure was subtracted from total ^{3}H dpm in all studies. Additional experiments to study the effect of osmolarity on membrane vesicle D-[^{3}H]glucose content was performed as described above except that varying concentrations (0.1–0.5 M) of raffinose were present in the incubation mixture and triplicate samples were taken at 15 min only.

Statistical methods. Student's *t*-test was used for statistical analysis of differences between experimental and respective control groups [40]; *P* values < 0.05 were considered statistically significant.

Results

Electron-microscopic appearance of the membrane preparations

Representative electron micrographs of membranes prepared by the present technique (preparation A) and the conventional technique (preparation B) are shown in Fig. 1. Preparation B consisted of vesicles, paired membrane sheets joined by junctional complexes, a partially disrupted canaliculi. Preparation A consisted almost exclusively of small vesicles without membrane sheets or junctional complexes. Few recognizable organelles were present in either preparation. Preparation A did contain glycogen part-

Fig. 1. Electron micrographs (X40 000) of liver plasma membranes prepared in the present technique (preparation A, A) and liver plasma membranes prepared by a conventional technique yielding a canaliculi-enriched preparation (preparation B, B). Whereas preparation A consisted almost exclusively of vesicles, preparation B contained tight junctions and paired membrane sheets as well as vesicles.

icles and rare, electron-dense, membrane-bound structures compatible with lysosomes.

Enzymatic profile and lipid composition of the membrane preparations

The enzymatic activities and lipid composition of preparations A and B are summarized in Table I. Preparation A differed significantly from preparation B in several respects. Mg^{2+}-ATPase specific activity was significantly greater in A than in B. However, because of the nearly 5-fold greater Mg^{2+}-ATPase activity in homogenate A compared with homogenate B, Mg^{2+}-ATPase enrichment compared to homogenate was actually less in preparation A than B. $(Na^+ + K^+)$-ATPase activity in homogenate A was four times greater than in homogenate B. Despite this, $(Na^+ + K^+)$-ATPase specific activity and enrichment compared to homogenate were markedly lower in preparation A than in preparation B. Deoxycholate (1 and 4 mM) produced a dose-dependent inhibition of $(Na^+ + K^+)$-ATPase activity when added to both preparation A (34% and 83% inhibition, respectively) and B (56% and 89% inhibition, respectively). Compared with preparation B, preparation A was significantly less contaminated with mitochondria, as assessed by succinate-cytochrome *c* reductase enrichment, and significantly more contaminated with microsomes, assessed by NADPH-cytochrome *c* reductase enrichment. Comparison of the NADPH-cytochrome *c* reductase specific activities in preparation A and a preparation of rat liver microsomes [41] indicated that about 15% of the total membranes present were accounted for by microsomes. Galactosyltransferase activity, which has been shown to be a marker of Golgi membranes [36], was greater in preparation A than in homogenate. Comparison of the galactosyltransferase activities in preparation A and purified Golgi membranes from rat liver [36] indicated that less than 5% of the total membranes were of Golgi origin. Enrichment and specific activity of alkaline phosphatase and 5′-nucleotidase were not significantly different in preparations A and B. In three experiments, no evidence of an $(Mg^{2+} + Na^+)$-stimulated, ouabain-resistant ATPase was found in

TABLE I

COMPARISON OF LIVER PLASMA MEMBRANE (LPM) A AND LPM B

Figures are presented as mean ± S.D.

		Yield	Lipid composition			Lipid fluidity
		mg LPM protein/g perfused liver	Cholesterol (μmol/mg protein)	Phospholipid (μmol/mg protein)	Cholesterol/ phospholipid molar ratio	Fluorescence polarization *P*
Prepn. A (n = 3–12)	LPM	0.42 ± 0.11	0.17 ± 0.03 **	0.46 ± 0.06 **	0.38 ± 0.06 **	0.280 ± 0.008 *
	Homogenate	–	–	–	–	–
	Enrichment	–	–	–	–	–
Prepn. B (n = 6–8)	LPM	0.55 ± 0.21	0.23 ± 0.02	1.08 ± 0.21	0.25 ± 0.04	0.288 ± 0.009
	Homogenate	–	–	–	–	–
	Enrichment	–	–	–	–	–
Renal/ intestinal microvillus membranes (Refs. 1, 3–7 and 9)	Enrichment (range)	0.9–2.3% of total homogenate	–	–	–	–

[a] *p*-Nitrophenol.

[b] Succinate- and NADPH-cytochrome *c* reductase activities were calculated in arbitrary units (ΔA/mg/min) and only the enrichment is therefore presented here.

preparation A or B studied either fresh or after storage in the cold for two weeks. Finally, compared with preparation B, preparation A had less cholesterol and phospholipid per mg protein, had a greater cholesterol to phospholipid molar ratio, and was significantly more fluid as reflected by a lower value for fluorescence polarization (*P*).

Segregation of marker enzymes

A balance sheet of marker enzyme activities during the isolation of membrane preparation A is outlined in Table II. Preliminary attempts to measure protein and enzyme activity in P_1 were frustrated by the presence of many clumps in the precipitate, presumably induced by the presence of Mg^{2+}. Recovery of measurable protein and enzyme activity in P_1 was approximately doubled by dispersion of the precipitate in 20 times the volume of buffer A to which 1 mM EDTA had been added, but was still probably incomplete, since recovery of protein and enzyme activity in S_1 and P_1 average only 87 ± 7% of that present in the homogenate (Table II). EDTA did not nonspecifically activate marker enzymes, thereby increasing apparent recovery, since measurable protein and enzyme activity in S_1 was unchanged (101 ± 11%) after identical 20-fold dilution in buffer A containing 1 mM EDTA.

It is clear from Table II that the initial low speed centrifugation in the presence of Mg^{2+} was primarily responsible for the separation of $(Na^+ + K^+)$-ATPase-bearing sinusoidal and lateral membranes as well as mitochondria and microsomes. That is, of total $(Na^+ + K^+)$-ATPase, succinate-cytochrome *c* reductase, and NADPH-cytochrome *c* reductase activity present in S_1 and P_1, 93%, 99%, and 62%, respectively, was recovered in P_1. It is also noteworthy that 74% and 53%, respectively, of Mg^{2+}-ATPase and alkaline phosphatase activity present in S_1 and P_1 was recovered in P_1 as well. It is therefore likely that the effects of Mg^{2+} were not entirely selective, and some canalicular membranes were also precipitated during the initial centrifugation.

Solute transport studies

A representative study of D-[^{3}H]glucose uptake by membrane vesicles is shown in Fig. 2. Uptake of

Enzyme activity						
Mg^{2+}-ATPase (μmol P_i mg/h)	$(Na^+ + K^+)$ ATPase (μmol P_i mg/h)	Alkaline phosphatase [a] (μmol pNP/mg/h)	5'-Nucleotidase (μmol P_i/mg/h)	Succinate-cytochrome *c* reductase	NADPH-cytochrome *c* reductase	Galactosyl-transferase (nmol galactose/mg/h)
113.1 ± 42.2 **	7.7 ± 5.8 **	6.7 ± 4.2	59.9 ± 23.1	– [b]	– [b]	4.4 ± 4.2
11.5 ± 5.6 **	2.9 ± 1.1 **	0.41 ± 0.26	3.2 ± 0.9	–	–	0.94 ± 0.34
10.9 ± 4.6 **	2.8 ± 1.7 **	20.5 ± 14.7	20.6 ± 11.4	0.07 ± 0.98 **	1.5 ± 0.3 **	4.3 ± 2.9
69.7 ± 27.3	23.0 ± 6.5	6.4 ± 1.4	63.3 ± 16.5	–	–	– [c]
3.3 ± 1.0	0.80 ± 0.46	0.25 ± 0.05	2.8 ± 0.6	–	–	–
22.3 ± 11.4	53.0 ± 55.5	27.8 ± 11.8	23.8 ± 8.6	0.21 ± 0.14	0.21 ± 0.06	–
–	0.1–2.2	7.8–23.0	–	–	0–0.5	–

[c] Galactosyltransferase activity was not measured in preparation B.

* $P < 0.05$.

** $P < 0.001$.

TABLE II

BALANCE SHEET OF MARKER ENZYME ACTIVITY DURING MEMBRANE PREPARATION

All figures represent mean ± S.D. of four to six liver plasma membrane A preprations. n.d., not detectable.

	Protein (mg)	Mg^{2+}-ATPase (μmol P_i/mg/h)	$(Na^+ + K^+)$-ATPase (μmol P_i/mg/h)	Alkaline phosphatase (μmol P_i/mg/h)	Succinate-cytochrome *c* reductase [a]	NADPH-cytochrome *c* reductase [a]
Homogenate (total)	1 269 ± 178	5 930 ± 1 443	3 461 ± 1 909	352 ± 108	–	–
	Percentage					
Homogenate	100	100	100	100	100	100
P_1 [b]	38.5 ± 8.9	67.8 ± 62.6	81.4 ± 18.8	39.4 ± 9.9	93.7 ± 50.8	54.2 ± 13.1
S_1	47.0 ± 6.0	24.1 ± 5.9	5.7 ± 8.4	34.5 ± 6.5	1.1 ± 2.2	33.4 ± 2.3
P_2	4.6 ± 4.2	13.6 ± 6.7	2.1 ± 4.4	19.4 ± 19.0	1.9 ± 2.7	15.2 ± 8.1
S_2	38.9 ± 6.5	8.2 ± 1.4	4.3 ± 2.5	15.3 ± 6.1	n.d.	26.3 ± 2.4
P_3	0.36 ± 0.06	2.0 ± 0.9	0.76 ± 0.60	2.40 ± 0.86	n.d.	1.6 ± 3.3
S_3	4.3 ± 0.4	8.0 ± 1.4	3.5 ± 3.7	14.4 ± 10.5	n.d.	23.8 ± 2.0
P_4	0.56 ± 0.02	5.1 ± 3.9	0.74 ± 0.93	7.1 ± 2.8	n.d.	1.8 ± 0.4
S_4	4.1 ± 0.77	1.1 ± 1.3	1.1 ± 1.6	3.4 ± 1.5	n.d.	23.0 ± 0.1
LPM	0.06 ± 0.02	1.7 ± 1.3	0.20 ± 0.13	1.6 ± 0.6	n.d.	0.09 ± 0.06

[a] Only relative specific activities calculated.

[b] P_1 was dispersed in 20 times the volume of homogenization buffer containing 1 mM EDTA prior to measurement of protein and enzyme activity.

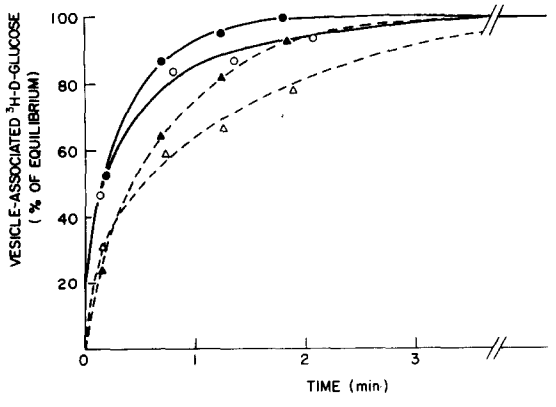

Fig. 2. Uptake of D-glucose by liver plasma membrane vesicles in the presence (broken lines) and absence (solid lines) of 100 μM phloretin. Membranes enriched in canaliculi prepared by a conventional technique (preparation B, open circles and triangles) and membranes prepared by the present technique (preparation A, closed circles and triangles) were incubated in a solution containing 100 mM NaCl, 87 mM mannitol, 0.87 mM Tris-Hepes (pH 7.5), and 1 mM D-[^{3}H]-glucose. Four aliquots were removed in the first three min and vesicles were separated from medium by suction filtration and triplicate samples were taken at 15 min (equilibrium).

D-glucose by vesicles from preparation A and preparation B was similar in all respects. One-half equilibrium values were observed in approx. 10–12 s, and equilibrium values were achieved within 3 min. Additional studies (not shown) indicated that the uptake rate of D-[^{3}H]glucose was not slowed by 50 mM unlabeled D-glucose compared with 1 mM D-glucose. D-[^{3}H]-Glucose uptake was the same in the presence and absence of a sodium gradient, and no overshoot suggestive of sodium cotransport [10,11] was observed. Uptake of D-[^{3}H]glucose was not inhibited by 10 μM phlorizin but was slowed by 10 μM phloretin ($t_{1/2}$ = 25–30 s) (Fig. 2).

D-[^{3}H]Glucose inside vesicles was distinguished from D-[^{3}H]glucose bound to membranes or present in adherent medium in two ways. First, D-[^{14}C]-glucose was present in the ice-cold stop solution. Thus, presuming that ^{14}C- and ^{3}H-labeled D-glucose were bound equally to membranes and present in equal concentrations in adherent medium after vortexing, the ^{14}C counts present on the filter could be used to determine D-[^{3}H]glucose associated with membranes but not inside vesicles (see Methods). In 40 studies,

an average of 91.8% of total D-[^{3}H]glucose associated with membranes was 'intravesicular'. All uptake curves including that shown in Fig. 2 represent intravesicular D-[^{3}H]glucose. Second, the effect of increasing medium osmolarity on intravesicular D-[^{3}H]glucose content at equilibrium was studied. As has been demonstrated for renal and intestinal brush border vesicles, increasing osmolarity with a relatively impermanent solute such as raffinose (M_r 594) would be expected to partially collapse the membrane vesicles, decrease the intravesicular space, and thus decrease intravesicular D-[^{3}H]glucose content at equilibrium. As summarized in Table III, increasing amounts of raffinose caused a profound and progressive fall in vesicle-associated D-[^{3}H]glucose.

Assuming that intravesicular D-glucose concentration at 'equilibrium' was equal to D-glucose concentration in the incubation medium, it was possible to calculate intravesicular volume. Intravesicular volume in the absence of raffinose ranged from 0.6–0.8 μl/mg protein and was not different for preparation A and B. The coefficient of variation of vesicle-associated D-[^{3}H]glucose (after correction for adherent glucose using D-[^{14}C]glucose) for triplicate samples taken at equilibrium (39 studies) averaged 6.9%.

TABLE III

EFFECTS OF OSMOLALITY ON EQUILIBRIUM CONTENT OF D-GLUCOSE IN LIVER PLASMA MEMBRANE VESICLES

Membrane preparations A and B were incubated in the standard incubation medium containing 1 mM D-[^{3}H]glucose to which varying amounts of raffinose had been added. Membranes were separated from medium by suction filtration. Each value represents the mean of three determinations at 15 min.

Raffinose (concn.) (mM)	D-Glucose content (pmol/mg)	
	Prepn. A	Prepn. B
0	810	897
100	417	356
200	381	315
333	336	280
500	281	279

Discussion

The technique used in this study for preparation of liver plasma membranes differs from conventional preparative techniques [13–30] in two major respects. First, instead of gentle tissue homogenization, e.g. by fifteen up-and-down strokes of a Dounce homogenizer in technique B, the liver parenchyma is vigorously disrupted by a 4 min homogenization in a Sorvall Omnimix. It is likely that this process, which more thoroughly fragments the plasma membrane than conventional techniques, may allow more complete separation of the individual fragments from different portions of the hepatocyte surface on the basis of physical properties such as density. Thus, preparation A is comprised of a relatively homogeneous collection of vesicles without the accompanying tight junctions and membrane sheets of probable non-canalicular origin found in preparation B. It is also apparent, however, that vigorous disruption makes electron-microscopic recognition of intact structures such as canaliculi, which previously has been used to ascertain membrane origin [14,19], impossible. Thus, identification of the origin of the membranes rests heavily on correlation of enzymatic activity with hepatocyte cytochemical studies. Vigorous tissue disruption also may account for the higher enzyme activities, particularly ATPase activity, in homogenate A than in B. That is, thorough fragmentation of the plasma membrane may expose increased numbers of enzyme active sites. The greater Mg^{2+}-ATPase activity in homogenate A than B may also have resulted in part from greater activation or release of non-plasma membrane (e.g. mitochondrial or microfilamentous/microtubular) Mg^{2+}-ATPase.

The second major difference in the current technique is the use of Ca^{2+} or Mg^{2+} during differential centrifugation. Ca^{2+}- and Mg^{2+}-induced aggregation of microsomal membranes has been recognized for some time [42] and has been used by several investigators to separate microsomes by low speed centrifugation [43,44]. Schmitz and co-workers [7] who recognized that Ca^{2+} aggregates not only microsomes but probably mitochondria and basolateral membranes as well, speculated that brush border membranes, despite their negatively charged glycocalyx, may fail to interact with divalent cations. Booth and Kenny, however, demonstrated that microvillus membranes readily

bind Mg^{2+} and suggested that the propensity of microvillus membranes to form aggregates may be selectively inhibited by divalent cations compared with other cell membranes [1]. Whatever the mechanism, use of Ca^{2+} or Mg^{2+} has greatly facilitated the preparation of intestinal and renal brush border microvillus membranes, and the present findings (Table II) confirm that precipitation of antiluminal membranes and microchondria as well as microsomes occurs during low speed centrifugation in the presence of Mg^{2+}.

Application of this preparative technique to rat liver yielded a preparation consisting predominantly of small vesicles by electron microscopy (Fig. 1). Although it is impossible to establish the origin of these membranes on the basis of morphology, electron micrographs failed to demonstrate contamination with recognizable intracellular organelles such as intact or disrupted mitochondria or rough endoplasmic reticulum, and dense membrane-bound bodies morphologically compatible with lysosomes only rarely were seen. The minimal activity of succinate cytochrome c reductase activity (Table I) in preparation A suggests trivial mitochondrial contamination, and comparison of the galactosyltransferase activity with that of purified Golgi membranes [36] indicates that Golgi membranes accounted for less than 5% of membranes in the preparation. Approximately 15% of the membranes in preparation A originated in the endoplasmic reticulum, as indicated by NADPH-cytochrome *c* reductase activity. These observations, plus the approximately 20-fold enrichment of the plasma membrane markers, 5′-nucleotidase and alkaline phosphate, indicate that the preparation consisted largely of liver plasma membranes. However, in contrast to preparation B and to other 'canaliculi-enriched' liver plasma membrane preparations currently used [19,24–28,32,45,46], preparation A is minimally enriched in $(Na^+ + K^+)$-ATPase compared with homogenate. This diminished activity and enrichment of $(Na^+ + K^+)$-ATPase activity does not result simply from destruction of the enzyme by the vigorous homogenization, since $(Na^+ + K^+)$-ATPase activity is actually 4-fold greater in homogenate A than B. Diminished $(Na^+ + K^+)$-ATPase activity also does not reflect a greater proportion of 'hidden' enzyme active sites in preparation A, since addition of the detergent deoxycholate decreased, rather than increased $(Na^+ + K^+)$-ATPase activity in both preparations. Comparison of the enzymatic profile of preparation A with recent cytochemical studies [47,48] localizing $(Na^+ + K^+)$-ATPase to the sinusoidal/lateral surface of the hepatocyte and alkaline phosphatase and Mg^{2+}-ATPase to the canalicular surface of the hepatocyte suggests that these mambranes are predominantly canalicular in origin. The observations that renal/intestinal brush border membranes and canalicular microvillus membranes (preparation A) can be isolated using the same technique and are similarly enriched in $(Na^+ + K^+)$-ATPase and alkaline phosphatase suggests also that these two types of luminal membranes are structurally and perhaps functionally alike, despite the fact that they originate from absorptive and secretory epithelia, respectively.

Comparison of preparation A with a conventional canaliculi-enriched preparation B reveals significant differences in composition and lipid fluidity as well as enzymatic activity (Table I). Presuming that preparations A and B differ primarily by the presence of tight junctions and sinusoidal or lateral membranes, then these results suggest that canalicular microvillus membranes have less cholesterol and phospholipid, a higher cholesterol: phospholipid molar ratio and greater lipid fluidity that sinusoidal and lateral membranes. These findings differ somewhat from those of Kremmer et al. [31] and Fisher et al. [23], who found that the lipid to protein weight ratio in their canalicular fraction tended to be higher than in their lateral and/or sinusoidal membrane fraction. It is thus likely that preparation A and the canalicular fraction of previous investigators [23,31] differ either in the amount of non-canalicular membranes present, or in that portion of the canalicular membrane represented. At present, the physiologic significance of the observed differences between preparations A and B in lipid composition and fluidity are uncertain. They do, however, indicate that hepatocyte luminal and antiluminal membranes differ in composition and physical properties as well as location and enzymatic activity.

Several lines of reasoning have suggested that an Na^+ pump other than $(Na^+ + K^+)$-ATPase might be operative in renal Na^+ transport [49], and evidence for an $(Mg^{2+} + Na^+)$-dependent ATPase in renal tissue has been reported in renal tissue stored in the cold for two weeks [34]. Because an Na^+ pump in canalicular

membranes conceivably might play a role in canalicular bile formation, we looked for evidence of an $(Mg^{2+} + Na^{+})$-stimulated ATPase in both liver plasma membrane preparation A and B. No such ATPase was found either in freshly isolated liver plasma membrane stored for two weeks in the cold.

Up to this point in the discussion, the canalicular membrane has been implicity treated as a homogeneous entity. It is possible, however, that regional differences in composition and enzyme activity are present within the canalicular membrane itself. In this regard, it is noteworthy that recovery of total homogenate alkaline phosphatase and Mg^{2+}-ATPase activity in preparation A was low (Table II). Since 63% of alkaline phosphatase activity [50] as well as a substantial portion of Mg^{2+}-ATPase activity [51] in liver is present in locations other than the plasma membrane, complete recovery of these canalicular marker enzymes in preparation A would not be expected. Nonetheless, the low recovery of these enzymes suggests that only a portion of the canalicular membrane is present in preparation A, and it is therefore conceivable that other membranes comprising the canaliculus may differ from preparation A.

Preliminary findings regarding solute movement into vesicles composed of liver plasma membranes are also reported. Study of solute movement into vesicles composed of luminal and antiluminal membranes has already proved a valuable tool in study of renal and intestinal transport [10,11]. The use of vesicles for this purpose not only facilitates characterization of the transport mechanisms operative at opposite ends of these polar cells, but it permits the role of the plasma membrane and transmembrane ionic gradients in equilibrative and concentrative transport processes to be studied independently of intracellular metabolism and binding. These are particular advantages in the study of hepatic solute transport.

Glucose was chosen for these preliminary studies because it does not present technical problems such as nonspecific binding to filters and/or membranes or self-aggregation which may occur with bile acids, bilirubin, and other organic anions. Vesicles were separated from the incubation medium by rapid suction filtration and D-[^{3}H]glucose inside vesicles was distinguished from D-[^{3}H]glucose present in adherent medium or bound to liver plasma membrane by the use of D-[^{14}C]glucose in the stop solution. The intravesicular location of D-[^{3}H]glucose was further established by demonstrating that increasing medium osmolarity, which presumably resulted in partial collapse of the vesicles, markedly decreased vesicle-associated D-[^{3}H]glucose. D-Glucose equilibrated across vesicles from both preparations A and B with a $t_{1/2}$ of approx. 10–12 s. No evidence of saturation was observed at D-glucose concentrations up to 50 mM, and D-glucose equilibration appeared to be slowed by the presence of 100 μM phloretin, but not by phlorizin. Phloretin and phlorizin are relatively selective inhibitors of carrier-mediated D-glucose uptake by mammalian cells via the Na^+-independent and Na^+-cotransport pahtways, respectively [52,53]. In the presence of an Na^+ gradient, D-glucose entry into the vesicles was not accelerated and no overshoot was observed. This is in contrast to previously reported findings for certain amino acids [29,30] and taurocholate [54], both of which have been shown to enter hepatocytes largely via an Na^+ coupled transport mechanism [55,56].

Limited information is available regarding glucose transport in vivo by the liver. Use of the multiple indicator dilution technique has indicated that D-glucose transport across the sinusoidal membrane is a carrier-mediated equilibrative process with a K_m exceeding 100 mM [57]. Although glucose entry can be inhibited by phlorizin, much higher concentrations (10^{-2} M) than those used here were necessary to achieve this effect [58]. Since it has not been possible to sample canalicular bile, no direct studies of glucose movement across the canalicular membrane have been performed. Indirect evidence, however, suggests that glucose does equilibrate rapidly between canalicular bile and liver cell water [59]. The preliminary studies reported here confirm rapid D-glucose movement across vesicles composed of canalicular membranes, but do not provide enough information to classify the process as carrier-mediated or simple diffusion. However, the observed $t_{1/2}$, lack of inhibition by phlorizin, and inhibition by phloretin are similar to the reported characteristics of the equilibrative, carrier-mediated D-glucose transport system present in the antiluminal (basolateral) plasma membranes of small intestine and renal brush border cells [53]. Since it is unclear whether vesicles in preparation B that took up glucose are of sinusoidal/

lateral or canalicular origin, it is impossible at this time to draw conclucions regarding the nature of glucose transport across sinusoidal/lateral liver plasma membranes in vitro.

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