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Characterization of Rat Liver Cell Plasma Membranes[1, 2] (38998)

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In part because of their structural heterogeneity and in part because methods for their isolation in sufficiently pure form have only recently become available (1), the plasma membranes of mammalian liver cells have not been studied extensively. There have been several reports on the protein and lipid composition of these membranes (2–8) but the reports have often involved insufficient ultrastructural control of the purity of the preparations, have involved the use of heterogeneous preparations, and have involved little effort directed towards structure-function relationships. Song *et al.* contributed an important step toward resolving the heterogeneity problem by reporting a method for the isolation of a liver cell plasma membrane fraction rich in intact bile canaliculi (9). Evans also reported the fractionation of rat liver cell plasma membranes into a low density vesicular and a high density sheet-like fraction (10). But neither of these reports involved those detailed chemical studies that will be necessary before the molecular events associated with the membrane transport processes can be understood. In this paper we report a modification of the methodology of Song *et al.*, which provides a pure fraction of the noncanalicular plasma membranes (PM) as well as of the bile canalicular membranes (BCM), and we report some of the chemical features of these membranes.

Materials and methods. Male Wistar rats (High Oak Ranch, Toronto, Ontario) weighing approximately 150 g were used. Prior to use the animals were kept in a constant temperature environment (22°), were in darkness 12 hr each day (7 PM to 7 AM) and were allowed water *ad libitum*. For at least 1 week prior to use they were maintained on a powdered semisynthetic diet (Teklad Mills, TD-72460, Basal Diet with 27% casein and salt mixture USP XIV). Separation of the BCM from the PM depends in part on differences in their lipid content and in order to standardize the nutritional status of the animals as much as possible they were force fed an amount of diet equal to 1 g/150 g body wt 2 hr immediately prior to sacrifice.

Liver cell plasma membranes were isolated according to a modification of the method of Song *et al.* (9). Each animal was anesthetized with diethyl ether and its liver was perfused via the portal vein with ice-cold physiological saline, pH 7.4 at 15 cm pressure. The liver was removed and weighed in a centrifuge tube (Sorvall) containing 10 ml of ice-cold 1 m*M* sodium bicarbonate, pH 7.5. Bicarbonate solution was added to a total volume in milliliters equal to twice the liver weight in grams and the liver was then homogenized using a Willems Polytron Homogenizer, at speed 7 for 5–10 sec. The homogenate was mixed with bicarbonate solution to a total volume of 100 ml and filtered twice, initially through four layers of cotton gauze, 20 × 12 (Texpack Ltd., Brantford, Ontario), and then through eight layers. The filtered homogenate was distributed among four tubes and centrifuged at 500*g* for 5 min and at 1000*g* for 10 min. The supernates were removed and unless required for the study of mitochondria and microsomes they were discarded. The pellets

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[2] The following abbreviations are used in this paper: BCM, isolated plasma membrane fraction consisting of bile canalicular membranes; PM, isolated plasma membrane fraction free from bile canalicular membranes.

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were combined and suspended in bicarbonate solution by gentle homogenization using a glass-Teflon homogenizer. After making the volume up to 100 ml the membrane suspension was centrifuged at 1000*g* for 10 min. The resulting pellets were combined and bicarbonate solution was added to a total volume in milliliters equal to the weight of the liver in grams. Following suspension by gentle homogenization a volume of sucrose solution of density 1.26, sufficient to give a final density of 1.22 was added. The volume of density 1.26 sucrose required is approximately 5.5 times the original liver weight in grams. After thorough mixing, 15-ml aliquots of the membrane suspensions, density 1.22, were put into ultracentrifuge tubes and overlayered with 9 ml of sucrose, density 1.18, and then with 3 ml of sucrose, density 1.16. After centrifugation at 66,000*g* (Rav) for 60 min using a Beckman 30 fixed angle rotor, membrane layers were observed at the 1.22–1.18 and at the 1.18–1.16 interfaces. The membrane layer at the 1.18–1.16 interface represented pure bile canalicular membranes. These membranes were harvested by syringe and needle and washed with 4 vol of bicarbonate solution. After centrifugation at 3000*g* for 10 min the membrane pellets were washed again in the same volume of bicarbonate solution. After a repeat centrifugation, the pellets were pooled, suspended in 30 ml of bicarbonate solution and centrifuged at 9000*g* for 10 min. The resultant pellet was the BCM (see Fig. 1). The membrane layer at the 1.22–1.18 interface represented the remainder of the plasma membranes but contained microsomes, mitochondria, collagen fibers, and occasionally even nuclei or the remnants of nuclei (see Fig. 2a). This layer was harvested by syringe and needle and the weight and volume of the aspirate documented. Sucrose, density 1.26, was added to give a final density of 1.22 using the following formula:

$$\text{Vol of d:1.26 sucrose} = \text{d:1.22} - \text{d:aspirate/0.04} \times \text{vol of membrane aspirate}$$

After thorough mixing 15-ml aliquots of the membrane suspension d:1.22 were overlayed with d:1.18 and d:1.16 sucrose as above and centrifuged at 66,000*g* for 60 min. The membrane layer at the 1.22–1.18 interface was harvested and submitted to the same washing procedure used for the BCM. The resulting pellet was the PM (see Fig. 2b).

For electron microscopic study, samples of the BCM and PM pellets were fixed for 1 hr at 0° in 2.5% gluteraldehyde buffered with 0.1 *M* cacodylate buffer, pH 7.4. After rinsing in the same buffer small blocks of the pellets were postfixed for 1 hr at 0° in 2% osmium tetroxide buffered with 0.1 *M* phosphate buffer, pH 7.4. They were dehydrated in a graded series of ethanol solutions and then embedded in a mixture of Epon 812 and Araldite. Ultrathin sections were stained with lead citrate and examined with a Philips EM-300.

Glucose-6-phosphatase, (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9), was measured by the method of Schwartz and Bodansky (11). 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) was measured by the method of Widnell and Unkeless (12). Leucyl-β-naphthylamidase, (L-leucyl-peptide hydrolase, EC 3.4.1.1.) was measured by the method of Goldbarg and Rutenberg (13).

Dry weights of the membrane subfractions were measured by drying each sample to a constant weight at 100°.

The protein content of the membrane suspensions was determined by the modified method of Lowry following solubilization in 0.1 *N* NaOH and using crystalline bovine serum albumin as standard (14).

Membrane preparations were homogenized for 2 min at room temperature in an atmosphere of nitrogen and 5 vol of chloroform–methanol, 2/1, v/v. After centrifugation for 10 min at 3000*g*, the organic phase was reserved and the above homogenization was repeated twice more. The three organic phases were pooled, partitioned against potassium chloride, dried over sodium sulphate, and concentrated under nitrogen at room temperature. Aliquots were taken for the following analyses: *Cholesterol* was determined by gas–liquid chromatography using a 2-ft column packed with 3% OV-1 and tridecanoin as internal standard. The

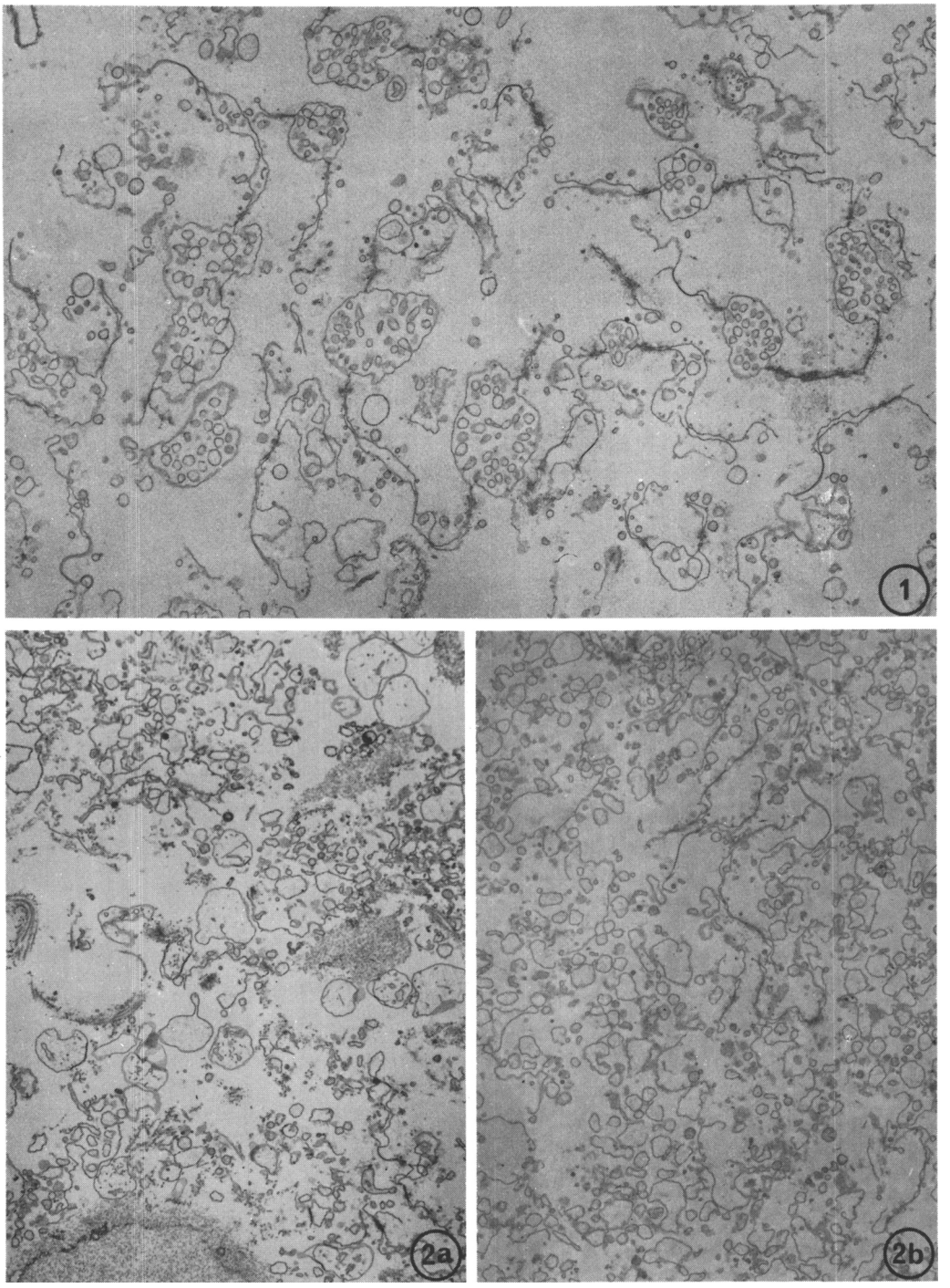

FIG. 1. Bile canalicular membrane pellet (BCM). Note the large number of intact bile canaliculi and the absence of contamination by other organelles. Lead citrate stain, × 7850.

FIG. 2a. Initial plasma membrane fraction (sucrose gradient 1.22–1.18 interface). Note the presence of many smooth membranes but also the contamination particularly by mitochondria, nuclear fragments. Lead citrate stain, × 5775. b. Final plasma membrane fraction (PM). This preparation is clean and comprised entirely of membranes. Lead citrate stain, × 5775.

temperature was 210–260° programmed at a rate of 10°/min. *Inorganic phosphorus* was measured by the method of Bartlett (15) after digestion of sample for 1 hr at 180° with 70% perchloric acid. Total phospholipid was calculated on the basis of 25 μg phospholipid/μg of lipid phosphorus. *Membrane phospholipids* were measured by applying an aliquot of the chloroform-methanol extract to a thin layer chromatographic plate coated with Silica Gel H 0.25–0.5 mm and separating them with a solvent system containing chloroform–methanol–water, 65/24/4. Bands corresponding to known phospholipid standards chromatographed simultaneously were eluted from the silica gel with chloroform–methanol–water–formic acid, 50/40/10/1, v/v, and their phosphorus content was measured using the method of Bartlett (15). *Total lipids* were determined by transferring an aliquot of the chloroform–methanol extract to a previously weighed aluminum cup and evaporating the sample under nitrogen and at low temperature to a constant weight.

Membrane porteins were analyzed by electrophoresis in polyacrylamide gels using the methodology of Fairbanks *et al.* (16).

Results. Examination by electron microscope of the material collected at the higher sucrose gradient interface revealed that the BCM fraction was composed of isolated bile canaliculi continuous with lateral hepatic plasma membranes interconnected by junctional complexes (see Fig. 1). The bile canaliculi in the BCM fraction were morphologically very similar to those in in situ liver tissue. The material collected from the lower interface (PM) consisted primarily of vesicles and sheets of plasma membrane. It was essentially free from bile canaliculi, and contained no other subcellular organelles (Fig. 2B).

Table I shows the specific activities of glucose-6-phosphatase, 5′-nucleotidase and leucyl-β-naphthylamidase activities in the membrane fractions. Glucose-6-phosphatase activity, normally confined to endoplasmic reticulum (17), was absent from the BCM and PM fractions, indicating that there was no measurable microsomal contamination of the BCM and PM. 5′-nucleotidase, an enzyme marker for plasma membrane in general was enriched over 20-fold in the PM (9, 18) and over 80-fold in the BCM fraction. Leucyl-β-naphthylamidase, suggested to be an enzyme characteristic of the bile canalicular membrane (19, 20) and not present in the rest of the plasma membranes in considerable amount was mainly located in the BCM fraction.

TABLE I. ENZYMATIC ACTIVITY OF THE PLASMA MEMBRANE FRACTIONS, MICROSOMES AND MITOCHONDRIA OF RAT LIVER RELATIVE TO TOTAL HOMOGENATE.

	Glucose-6-phosphatase	5′-nucleotidase	Leucyl-β-naphthylamidase
Whole homogenate	1.00 (94±2.9)[a]	1.00 (9±0.4)[a]	1.00 (5±0.3)[b]
Microsomes[c]	3.31	2.00	1.40
Mitochondria[c]	1.35	1.33	1.20
BCM	None	83.33	16.40
PM	None	23.78	1.00

Each value is from 12 determinations.

[a] Actual value, μmoles of phosphate released/mg protein/hr, at 37° (mean ± standard error).

[b] Actual value, μg β-naphthylamine liberated/mg protein/hr, at 37° (mean ± standard error).

[c] Microsomes were prepared by the method of Roberts *et al.* (32) and mitochondria by the method of Bock *et al.* (33).

The plasma membranes collected from both interfaces of the sucrose gradient (BCM plus PM) averaged 1.518 ± 0.046 (SEM) mg dry weight/gm of fresh liver tissue. Assuming a membrane thickness of 100 Å (3, 21), the theoretical yield from 1.00 cm^3 of liver (1.06 g) should be 2.85 mm^3 of membrane volume or 3.3 mg dry weight membrane material. The yield obtained by this method therefore averaged 46% of the theoretical yield. The recovery of 5′-nucleotidase activity in BCM + PM was about 20% of the total enzyme activity in the liver, and that of leucyl-β-naphthylamidase was only 5%, considerably lower values. This is probably due to their presence in subcellular elements other than BCM and PM (22, and see Table I), and perhaps to loss of activity during the isolation procedures. Studies in progress have confirmed losses *in vitro* when membrane preparations were

TABLE II. CHEMICAL COMPOSITION OF THE PLASMA MEMBRANE FRACTIONS OF RAT LIVER.

	PM	BCM	P^a
Dry weight[b, d]	1.029 ± 0.036	0.489 ± 0.030	<.01
Protein[b, d]	0.729 ± 0.027	0.264 ± 0.017	<.01
Lipid[b, d]	0.233 ± 0.019	0.189 ± 0.018	NS
Lipid[b, e]	0.320 ± 0.015	0.717 ± 0.050	<.01
Phospholipid[c, f]	0.267 ± 0.040	0.727 ± 0.107	<.01
Cholesterol[c, f]	0.176 ± 0.018	0.266 ± 0.023	<.02
Phosphatidyl choline[c, g]	30.1 ± 1.4	31.7 ± 1.7	NS
Phosphatidyl ethanolamine[c, g]	23.3 ± 2.1	22.6 ± 1.1	NS
Phosphatidyl serine plus phosphatidyl inositol[c, g]	22.3 ± 1.1	25.0 ± 1.3	NS
Sphingomyelin[c, g]	20.0 ± 1.3	17.7 ± 1.8	NS
Lysophosphatidyl choline[c, g]	4.3 ± 0.03	3.0 ± 0.1	<.01

[a] P value of Student's t test; NS, $P > .10$.
[b] 36 determinations.
[c] 12 determinations.
[d] mg/g liver.
[e] mg/mg protein.
[f] μmole/mg protein.
[g] Percentage of total phospholipids.

incubated in bicarbonate buffer (unpublished observation).

Table II shows the chemical analysis of the plasma membrane fractions. It is apparent that the two subfractions had widely different composition. The BCM which was recovered accounted for 32% of the total weight of plasma membrane isolated. This is in agreement with Evans *et al.* (7) who calculated that the light fraction rich in bile canaliculi accounted for 30% of the total plasma membranes recovered.

When calculated per unit weight of protein, there was more than twice as much total lipid in the BCM as in the PM fraction (0.717 ± 0.050 mg/mg protein in the BCM compared with 0.320 ± 0.015 mg/mg protein in PM). This could explain the widely differing densities of the two fractions as revealed in the sucrose gradients. The concentrations of total phospholipid and cholesterol were also higher in the BCM (0.727 ± 0.107 μmoles of phospholipid/mg of protein compared with 0.267 ± 0.040 μmole/mg; and 0.266 ± 0.023 μmole of cholesterol/mg of protein compared with 0.176 ± 0.018 μmole/mg). However, the composition of the phospholipids was similar in both fractions, phosphatidyl choline accounting for 31.7% and 30.0%, respectively, of the total in the BCM and PM fractions, phosphatidyl ethanolamine 22.6% and 23.3%, phosphatidyl serine plus phosphatidyl inositol 25.0% and 22.3%, and sphingomyelin 17.7% and 20.0%. On the other hand, the proportion of lysophosphatidyl choline was slightly but significantly lower in BCM, 3.0% compared with 4.3% ($P < .01$) in PM (see Table II).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate resolved the protein components of the membrane fractions into more than 20 bands and differences could be seen between the BCM and the PM. Examples of these electrophoretograms are shown in Fig. 3. Both fractions contained peptides with apparent molecular weights ranging from 10×10^3 to 270×10^3 (16). Both membranes contained proteins, mostly of molecular weight less than 160×10^3, which were easily solubilized in bicarbonate buffer. Studies to determine the nature of the differing proteins are being carried out.

Discussion. The plasma membrane of hepatocytes is not a uniform structure. It includes specialized areas such as bile canaliculi, desmosomes, tight junctions and the sinusoidal surface with its numerous microvilli (23). Subfractions of different regions show differences in density (9, 10). Song *et al.* (9) obtained a plasma membrane fraction rich in bile canalicular membranes.

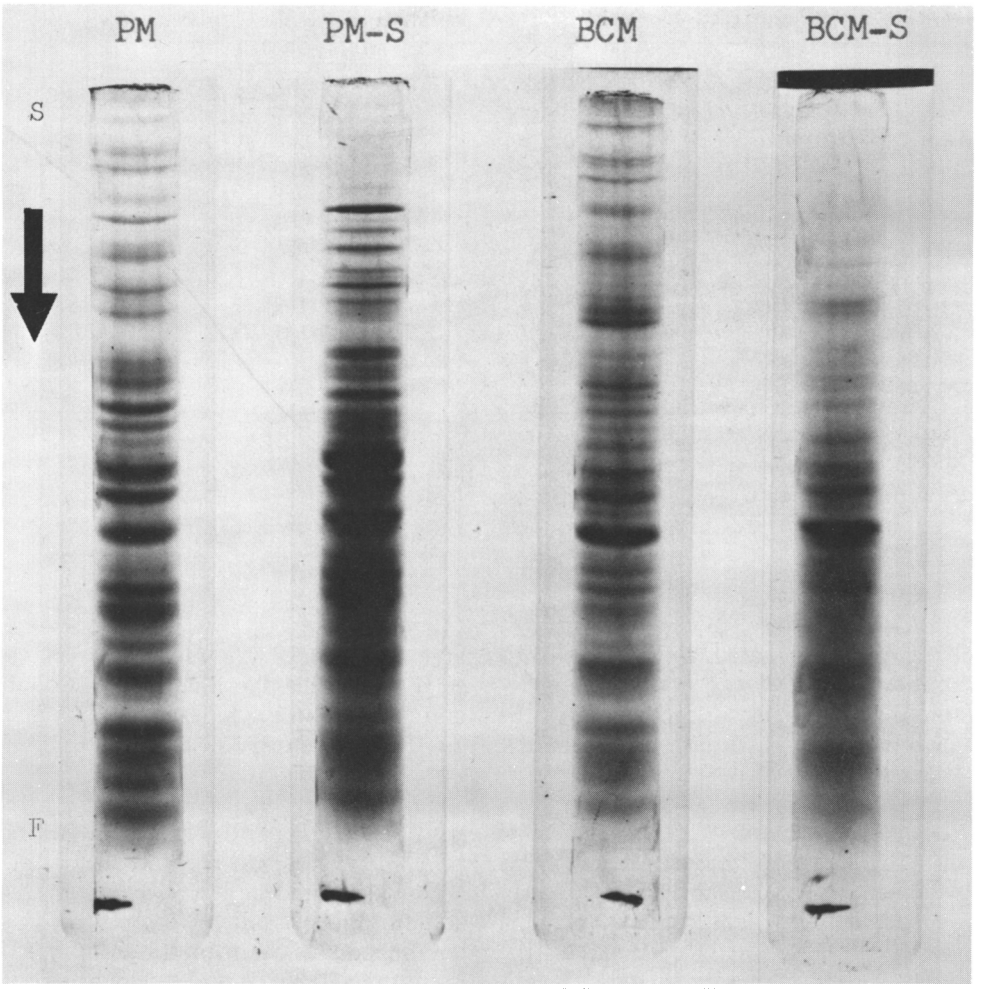

FIG. 3. Comparison of proteins of plasma membrane subfractions in the polyacrylamide SDS system. Approximately 100 μg of protein were applied to all the gels. The direction of the electrophoretic migration (towards the anode) is indicated by the arrow. PM, canaliculus-free plasma membrane. PM-S, soluble proteins from the PM in low ionic solution of sodium chloride 0.15%. BCM, bile canalicular membrane. BCM-S, soluble proteins from the BCM in low ionic solution of sodium chloride 0.15%. The average molecular weight of the fastest moving bands (F) is 10,000 and of the slowest moving bands (S) is 275,000.

This paper reports an improvement of this method, by which the remaining plasma membrane (bile-canaliculus-free) is also obtained in pure form. This allows comparative studies of the functionally different parts of the plasma membrane, hitherto not possible. The additional sucrose density gradient centrifugation of the bile canaliculi-poor fraction (1.22–1.18 sucrose interface layer; see Materials and methods) effectively removed contamination by microsomes, mitochondria and collagen fibers (Figs. 2a and 2b). Use of the Polytron homogenizer to break the cells increased the reproducibility of the results (see Materials and methods).

Electron microscopic examination of this, PM, fraction as well as of the bile canalicular membrane (BCM) fraction showed that they are both very pure. The enzyme measurements support this contention. The activity of the microsomal enzyme (17, 24) glucose-6-phosphatase was virtually absent from both BCM and PM, indicating no measur-

able contamination by microsomes. Leucine amino-peptidase activity, thought to be characteristic of plasma membranes (19, 20) was largely confined to the BCM indicating a considerable degree of purification of BCM. 5′-nucleotidase activity, similarly suggested to be a marker for plasma membranes (9, 18) was highly concentrated in both BCM and PM fractions.

The yield of plasma membranes obtained in these studies, averaging 46% of theoretical (see Results), was relatively high. Weibel *et al.* (25) estimated a yield of 15%, Lauter *et al.* (26) 14%, and Yunghans *et al.* (27) 7–20%. Other investigators have obtained a complete recovery of the membrane (compared with theoretical), but the degree of microsomal contamination was rather high (28). The reasons for these large differences in yield are not yet clear (28).

The chemical and electrophoretic analyses show that membrane fractions have widely differing composition with respect to both lipid and protein. Although the phospholipid composition of the two fractions was similar, other studies from this laboratory (29, 30) have shown marked differences in phospholipid biosynthesis, and in the activities of the enzymes concerned with phospholipid synthesis in these membranes (29, 31).

The very different structural and biochemical properties of the two plasma membrane fractions studied are in accord with the fact that they have different physiological functions. How these properties relate to their functions remains to be answered.

Summary. A method is described by which bile canalicular membranes (BCM) can be prepared, together with canaliculus-free plasma membrane (PM), both essentially free of contamination. The recovery of both fractions together was estimated to be 46%. The concentrations of total lipid, total phospholipid and cholesterol were substantially greater in the BCM, and polyacrylamide gel electrophoresis revealed differences in protein composition. The differences in lipid and protein composition of these two plasma membrane fractions are presumably related to their very different physiological functions.

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