PLASMA MEMBRANES OF THE RAT LIVER

Isolation and Enzymatic Characterization of a Fraction Rich in Bile Canaliculi

C. S. SONG, W. RUBIN, A. B. RIFKIND, and A. KAPPAS

From The Rockefeller University and the Departments of Medicine and Anatomy, Cornell University Medical College, New York 10021

ABSTRACT

A method is described for the rapid isolation of a plasma membrane fraction containing a high concentration of intact bile canaliculi from the rat liver. Isolated bile canaliculi retain most of the ultrastructural features exhibited in the intact liver cell. The final fraction contains 5′-nucleotidase activity at approximately the same concentration as that in previous preparations of plasma membranes. In the presence of 0.01 M Mg^{++}, 5′-nucleotidase exhibits a double pH optimum at pH values of 7.5 and 9.5. The activities of glucose-6-phosphatase and alkaline phosphatase are present in low amounts. Cytochrome P-450 is not detectable. Na^{+}-K^{+}-activation of ATPase is observed to the extent of 20–36% in about half of the assays. The availability of a method for preparation of intact bile canaliculi should prove useful for studying the biochemical events associated with the transport of bile constituents into canaliculi.

INTRODUCTION

In the vertebrate liver, plasma membranes of adjoining parenchymal cells make up bile canaliculi (tubular structures with villous projections of membranes) which are readily identifiable by means of electron microscopy (15). Bile canaliculi are the most proximal intrahepatic loci where various biliary constituents are transported from the parenchymal cells into the bile. Some of these substances, e.g. bilirubin and bile acids, are transported against a large concentration gradient (6); and such active transport of biliary components for eventual excretion into the intestinal tract constitutes one of the major functions of the liver. Isolation of bile canaliculi would therefore offer an excellent means of studying biochemical aspects of the transport phenomena associated with bile formation. We describe in this paper a rapid method for preparing a plasma membrane fraction from rat liver which contains relatively intact bile canaliculi in a good yield. Certain enzymatic properties of the isolated fraction have also been studied and compared with those of the whole homogenate and microsomal fraction of the rat liver.

MATERIALS AND METHODS

Materials

Water doubly distilled from glass was used throughout the experimental procedure. The following substrates were employed in various enzyme assays: AMP from Schwarz Bio Research Inc. (Orangeburg, N.Y.), β-glycerophosphate from Fisher Scientific Company (Pittsburgh, Pa.), and glucose-6-phosphate and Tris-ATP from Sigma Chemical Co. (St.

Louis, Mo.). Sodium citrate (Fisher), sodium diethylbarbiturate (Merck Chemical Div., Rahway, N.J.), or Tris (Sigma) was used as buffer in most experiments. Other chemicals were of reagent grade or of the highest quality available.

In all experiments we used female Sprague-Dawley rats (Charles River Breeding Laboratories, North Wilmington, Mass.) which weighed 150–200 g. They were allowed free access to food and water.

Chemical Analyses

Phosphorus was determined according to the method of Fiske and SubbaRow (16). Protein was measured with Phenol Reagent (Fisher) according to the method of Lowry et al. (20).

Determination of Enzyme Activity

The activities of 5′-nucleotidase (EC[1] 3.1.3.5.), alkaline phosphatase (EC 3.1.3.1.), glucose-6-phosphatase (EC 3.1.3.9.), and ATPase (EC 3.6.1.4.) were measured by determining the initial rate of release of inorganic phosphate from appropriate substrates at 37°, and the velocities were expressed as micrograms of phosphorus released per minute per milliliter reaction mixture. The assay for 5′-nucleotidase (4) was at pH 7.5 in the presence of 0.005 M AMP and 0.01 M Mg^{++}, and the assay for alkaline phosphatase (3) was at pH 9.1 in the presence of 0.01 M β-glycerophosphate and Mg^{++}. Glucose-6-phosphatase assay (29) was at pH 6.5 with 0.015 M glucose-6-phosphate as subtrate. Mg^{++}-ATPase assay, as described by Emmelot and Bos (11), was at pH 7.4 in the presence of 0.01 M Tris-ATP, 0.1 M KCl, and 0.005 M Mg^{++}. For determining Na^{+}-K^{+}-activated ATPase (11), 0.066 M NaCl and 0.034 M KCl were substituted for 0.1 M KCl, and the increment in the amount of P_i released was measured. Cytochrome P-450 was determined by measuring the CO-difference spectra (Cary Model 15 spectrophotometer) of $Na_2S_2O_4$-reduced preparations of plasma membrane fractions according to the method of Omura and Sato (24).

Isolation of Bile Canaliculi

Rats weighing 150–200 g gave the best yields. The animals were decapitated, and the livers were transferred to a beaker chilled in ice. All subsequent procedures were carried out at 0°–4°C. The livers were minced and placed in large Dounce homogenizers (Blaessig Glass Specialties, Rochester, N. Y.) with loose-fitting pestles. 2 volumes of 0.001 M $NaHCO_3$ buffer (pH 7.5) were added to approximately 10 g of minced liver in each homogenizer, and the mixture was gently homogenized by 30 strokes of the pestle. The homogenate was diluted with 250 ml of the same buffer, passed twice through four layers of surgical gauze, and then centrifuged for 10 min at 1,500 *g*. The pink, gelatinous pellets that formed in the bottom of the centrifuge bottles were pooled and suspended by gentle shaking in 0.001 M $NaHCO_3$ buffer to the final volume (milli/liters) corresponding to the original wet weight of the liver (grams). To this suspension of crude membranes, we added exactly 5.5 volumes of sucrose solution of density 1.26 (70.74%, w/v) and gently mixed the suspension in the Dounce homogenizer until the membranes became evenly dispersed. This required 2–10 strokes of the loose-fitting pestle. We took care not to extend the mixing beyond homogeneous suspension, since we noted that excessive homogenization at this point reduced the yield of isolated bile canaliculi. The density of the final suspension (1.22) was checked by weighing a 10 ml aliquot, and 15–16-ml aliquots of the suspension (designated M-1) were placed in cellulose nitrate tubes (Beckman, No. 302237). 7 and 5 ml of sucrose solutions of densities 1.18 (48.45%, w/v) and 1.16 (42.9%, w/v), respectively, were carefully layered in succession over M-1. The tubes were then placed in a Spinco No. 30 rotor and centrifuged for 60 min at 66,000 *g*.

Fig. 1 shows the appearance of a tube following centrifugation. Plasma membrane fractions present in M-1 floated up during centrifugation and accumulated at the two sucrose interfaces (*A* and *B*). Preliminary assay of materials from both interfaces (Fig. 2) for 5′-nucleotidase, a marker enzyme for plasma membrane (27, 33), showed a greater specific activity in fractions collected from the upper (*A*) interface. In all subsequent experiments, the grayish-white sheet of material from the upper interface in each tube was collected by means of a syringe, mixed with 4 volumes of 0.001 M $NaHCO_3$ buffer, and centrifuged for 10 min at 1,500 *g*. The resulting pellet was washed once by gentle suspension in about 20 volumes of the same buffer and recentrifuged for 10 min at 1,500 *g*. The final pellet (designated M-2), resuspended in distilled water or in 0.001 M $NaHCO_3$ buffer, was used in all enzymatic studies. The entire preparative procedure took approximately 3 hr for a single worker. Up to 30 g of pooled liver could be processed in one operation, yielding 4–6 mg of protein in M-2. M-2 yielded approximately 3% of total 5′-nucleotidase present in the initial homogenate.

Isolation of Microsomal Fraction

The method of Palade and Siekevitz (25) was used to prepare microsomal fractions from rat liver homogenates.

[1] EC, Enzyme Commission.

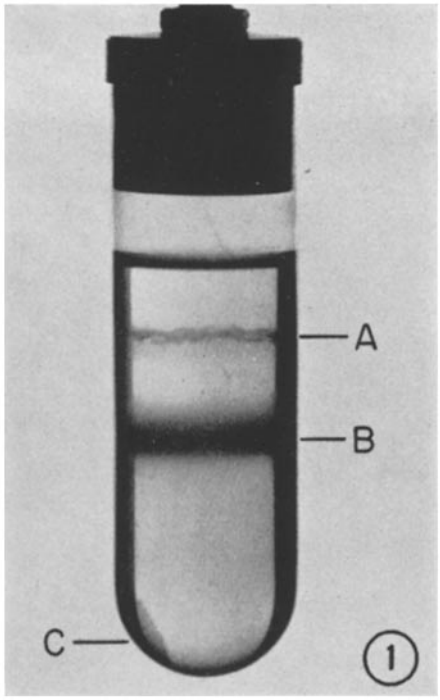


FIGURE 1 Centrifugation of crude preparation of plasma membranes (M-1) through discontinuous density-gradient solution of sucrose. 15 ml of M-1 prepared as described in the text (density 1.22) were placed in a cellulose nitrate tube and overlaid with 7 and 5 ml of sucrose solutions of densities 1.18 and 1.16, respectively. The tube was photographed after it had been centrifuged at 66,000 *g* for 60 min. The subcellular components present in M-1 separated into three fractions according to their buoyant density: grayish-white fraction at upper interface, *A*; light-brown fraction at lower interface, *B*; and pink pellet in the bottom of the tube, *C*.

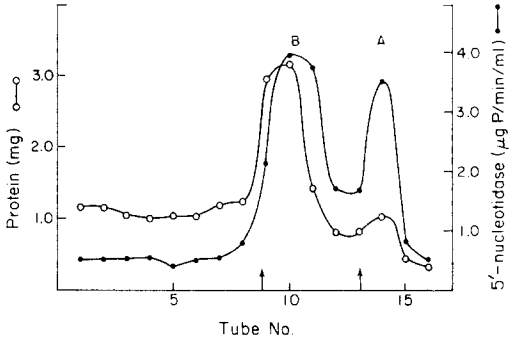


FIGURE 2 Assay of plasma membrane fractions separated by flotation through discontinuous density-gradient solution of sucrose. A sucrose gradient tube containing M-1 was prepared as described in the legend for Fig. 1 and was centrifuged at 66,000 *g* for 60 min. At the end of centrifugation, the bottom of the tube was punctured without disturbing the pellet, and 1.7-ml fractions were collected and assayed for protein (○—○) and 5′-nucleotidase (●—●). The effluent fractions are numbered from the bottom of the tube. The arrows indicate the interfaces of sucrose solutions. The specific activities of the fractions from the upper (*A*) and the lower (*B*) interfaces for 5′-nucleotidase were, respectively, 3.46 and 1.27 u/mg protein. The unit of 5′-nucleotidase activity is defined as micrograms phosphorus released per minute per milliliter reaction mixture.

Electron Microscopy

Pellets of tissue fractions were fixed in 2.5% (w/v) glutaraldehyde-0.1 M phosphate buffer (pH 7.4), dehydrated in graded concentrations of ethanol and propylene oxide, and embedded in Epon 812 (21). Thin sections were stained with lead citrate (34). All specimens were examined and photographed in a Philips EM-200 electron microscope.

RESULTS

Upon examination in the electron microscope, M-2, the material from the upper interface in the sucrose gradient tube (Fig. 1, *A*), showed a collection of bile canaliculi and smooth membrane vesicles (Fig. 3). The bile canaliculi, which represent only a small portion of the surface area of the hepatic cells (9, 18), were highly concentrated in M-2 (Fig. 4) and, except for slight swelling, exhibited little morphological difference from their original appearance *in situ* in hepatic parenchymal cells (15). The fibrillar cytoplasm which surrounds the canaliculi *in situ* (2) and extends into their microvilli was largely removed during the preparation, although a small amount remained adherent to the junctional complexes (2, 14, 18) immediately adjacent to the canaliculi (*I* and *D*, Fig. 4). In rare sections, we could see fragments of what appeared to be chromatin material and a few membrane fragments with attached ribosomes. In all sections, however, bile canaliculi were the predominant structural elements.

The material collected from the lower interface in the sucrose gradient tube (Fig. 1, *B*) contained, in addition to bile canaliculi and other fragments of plasma membranes, many mitochondria and membrane vesicles with attached ribosomes (Fig.

5); this finding is consistent with the lower specific activity for 5′-nucleotidase (Fig. 2) exhibited by this material. The amount of cell components present in the lower interface (Fig. 1, *B*), when compared to that in the upper interface (Fig. 1, *A*), was very much greater as was evident from the apparent turbidity and the protein content (Fig. 2) of the interfaces. At the end of centrifugation, therefore, a large number of bile canaliculi and fragments of plasma membrane were still retained in the lower interface of the gradient tube together with other cell components of higher density; this explains the greater total activity of 5′-nucleotidase in the material collected from the lower interface (Fig. 2). The pellet in the sucrose gradient tube (Fig. 1, *C*) consisted of fragments of nuclei, mitochondria, and erythrocyte ghosts (not depicted).

The enzymatic properties of the plasma membrane fraction (M-2) containing bile canaliculi isolated by the present method are summarized and compared with those of the microsomal fraction in Table I. 5′-Nucleotidase, a phosphohydrolase concentrated primarily in the plasma membrane and specifically in membranes forming bile canaliculi in the rat liver (27, 33), was approximately as active as in previously reported preparations of plasma membranes (13, 33). This may indicate that our preparation M-2, although highly concentrated in *intact* bile canaliculi, is probably not any more enriched than were previous preparations of plasma membranes in relative proportion of canalicular membrane to noncanalicular portion of plasma membrane. The specific activity of 5′-nucleotidase in M-2, however, was more than 10 times as high as that in the microsomal fraction of the liver (Table I). It had an optimum pH near 7.5 (Fig. 6). In the presence of 0.01 M Mg^{++}, a second pH optimum appeared near pH 9.5 due to marked activation of the enzyme activity. Nonspecific alkaline phosphatase activity was negligible in comparison to that of 5′-nucleotidase at this pH value.

TABLE I

Enzymatic Activities of Plasma Membrane Fraction (M-2) Containing Bile Canaliculi and Microsomal Fraction of the Rat Liver

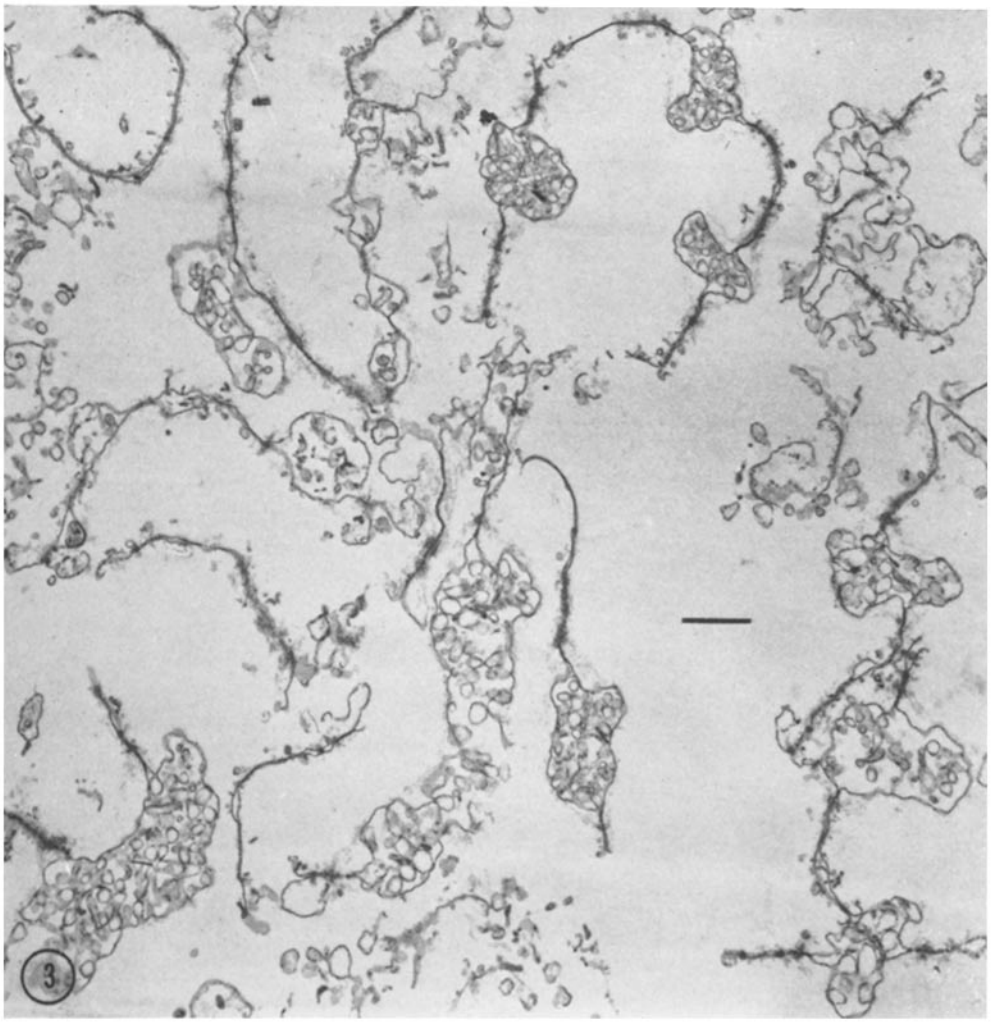
All enzyme activities were measured by determining the initial rate of release of P_i from appropriate substrates at 37° as described in the text. Each reaction mixture contained, in a total volume of 5 ml, 20–50 μg in protein of M-2 or 0.5–2.5 mg in protein of microsomal fraction per milliliter. Units of enzyme activities are defined as micrograms of phosphorus released per minute per milliliter reaction mixture. Specific activities are defined as units per milligram protein. The figures in parentheses indicate the number of experiments.

Enzymes	Specific activity in M-2	Specific activity in microsomal fraction
5′-Nucleotidase	4.65 (7)	0.44 (7)
Glucose-6-phosphatase	0.25 (5)	0.48 (7)
Mg^{++}-ATPase	5.32 (6)	0.72 (4)
Alkaline phosphatase	0.07 (6)	0.02 (7)

Glucose-6-phosphatase, an enzyme concentrated in the endoplasmic reticulum in the rat liver, was present in detectable amounts in M-2. Its specific activity, however, was only approximately half of that found in the microsomal fraction and was only slightly higher than that found in plasma membrane preparations previously reported (13, 33). Cytochrome P-450, a hemoprotein believed to be present in the endoplasmic reticulum of the rat liver (17, 24), was not detectable in M-2.

Mg^{++}-ATPase activity of M-2 was as high as reported values for plasma membranes (11, 13). Its specific activity was more than seven times as high as that in the microsomal fraction (Table I); this is consistent with histochemical evidence for localization of Mg^{++}-ATPase in the plasma membranes (23, 27). Na^+-K^+-activation of ATPase, attributable to an enzyme located in the surface membranes of a number of cell types (5, 26, 30) and believed to be intimately related to active ion transport (31), was not demonstrable with consistency; and its activity, in comparison to Mg^{++}-ATPase, was low where present. In approximately half of the assays, 20–36% activiation of ATPase by Na^+ and K^+ was noted.

Weak activity for alkaline phosphatase with β-glycerophosphate as substrate was observed in M-2. This was still significantly higher than the trace activity found in the microsomal fraction (Table I). In plasma membrane preparations previously reported (10, 13, 33), no alkaline phosphatase activity could be detected with β-glycerophosphate as substrate. Since weak activity of this enzyme can be localized to bile canaliculi by histo-

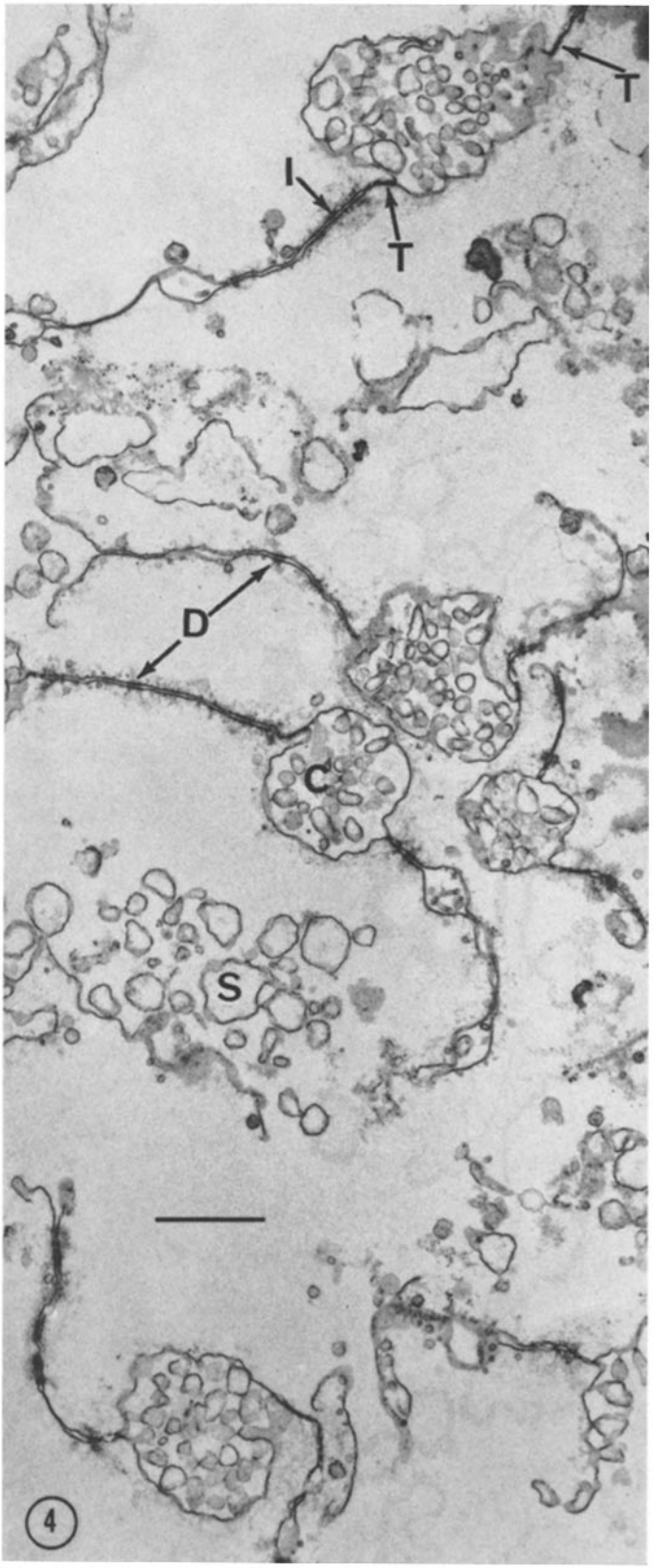


FIGURES 3 and 4 Electron micrographs of plasma membrane fraction (M-2) collected from the upper interface (*A*, Fig. 1) of discontinuous density-gradient solution of sucrose. Thin sections of M-2 were prepared as described in the text and were stained with lead citrate. Fig. 3, × 8,400. All scale lines are 1 μ.
FIGURE 4 See legend under Fig. 3. Note the smooth membrane vesicles (*S*) and the intact bile canaliculi (*C*) with junctional complexes of adjoining plasma membranes consisting of tight junctions (*T*), intermediate junctions (*I*), and desmosomes (*D*). × 15,000.

chemical means, our finding is compatible with the high concentration of intact bile canaliculi present in M-2. Part of the alkaline phosphatase activity in M-2 may also be attributable to the bile retained within the isolated canaliculi.

DISCUSSION

Methods for the general isolation of plasma membranes of the rat liver have been introduced by Neville (22) and Emmelot et al. (13). These procedures require extensive and time-consuming homogenizations alternated with centrifugations prior to final flotation through sucrose solutions. Moreover, they yield greatly fragmented membranes (1) containing no bile canaliculi or dilated and distorted bile canaliculi (8, 13, 22) in small yields (13, 33), and they are therefore not suitable for rapid preparation of plasma membrane fraction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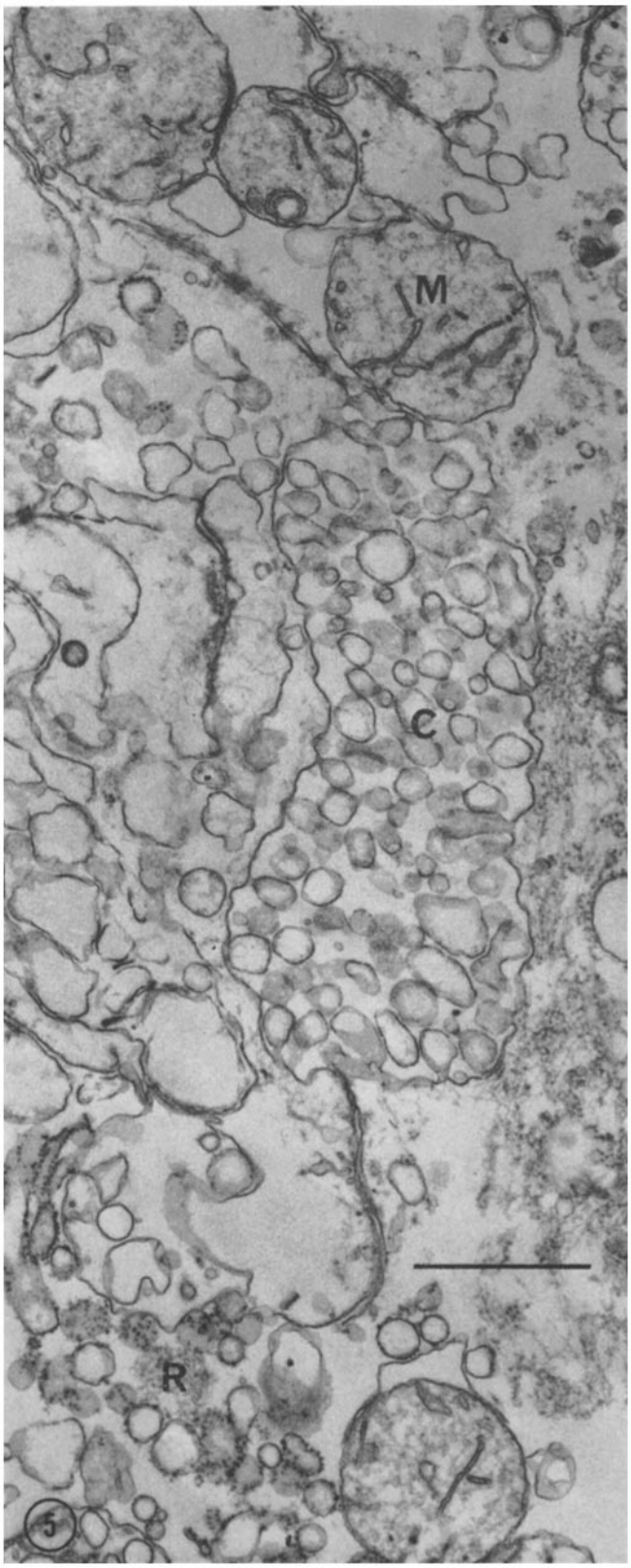

FIGURE 5 Electron micrograph of tissue fraction collected from the lower interface (*B*, Fig. 1) of discontinuous density-gradient solution of sucrose. The tissue fraction was removed by means of a syringe, diluted with 10 volumes of distilled water, and centrifuged at 76,000 *g* for 30 min. The pellet was fixed, and thin sections were prepared as described in the text. A bile canaliculus (*C*), mitochondria (*M*), and membrane vesicles with attached ribosomes (*R*) are present. × 24,000.

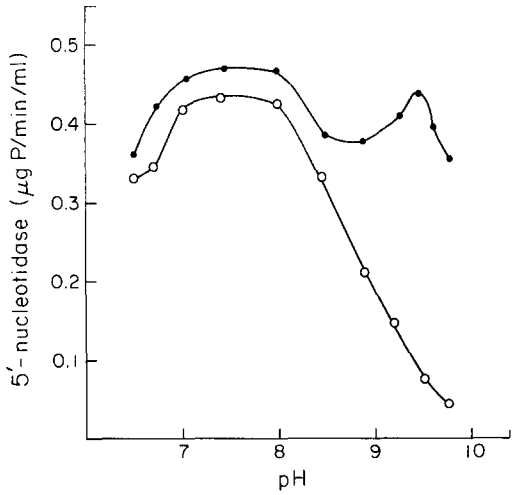

FIGURE 6 Effect of pH on the activity of 5'-nucleotidase of plasma membrane fraction (M-2) containing bile canaliculi. Final concentrations of the reactants were: AMP, 0.005 M; Tris-HCl, 0.1 M; and 20 μg in protein of M-2/ml reaction mixture. Initial velocities were measured in the indicated pH range in the presence (●—●) and in the absence (○—○) of 0.01 M Mg^{++}.

containing bile canaliculi morphologically resembling their native state. It has proved possible in the present studies to eliminate the initial series of differential centrifugations which most likely damage isolated bile canaliculi. In addition, we have introduced the use of a large-capacity fixed-angle rotor for the flotation step. These modifications minimize trauma to isolated bile canaliculi and substantially increase the final yield of the plasma membranes, while they reduce the total expenditure of time by approximately one half. Our procedure has consistently yielded relatively high concentrations of intact bile canaliculi, and the rapidity and the simplicity of this method should prove to be of value in studies of hepatic plasma membranes and bile canaliculi and especially in studies of biochemical mechanisms involved in the transport of bile constituents into bile canaliculi.

Light microscopists have long known that when liver tissue is crushed under a cover slip or when it is macerated and left in water for several days, the hepatocytes become totally destroyed, while networks of bile canaliculi remain intact (9). These observations supported the erroneous concept, held prior to electron microscopy, that the canaliculi represented minute tubules with their own solid stiff walls. Subsequent electron microscopic studies (2, 7, 14, 15, 28, 35), however, have shown the canaliculi to represent the glandular lumina of adjoining hepatocytes. The canalicular walls, which are resistant to disruption, are composed of the specialized surfaces of these hepatocytes and the junctional complexes (14) formed between them. The cytoplasm immediately adjacent to the canalicular surfaces (pericanalicular ectoplasm) and the junctional complexes of the adjoining cell membranes have been thought to play a major role in maintaining the integrity of the canalicular wall (2, 7, 14, 18, 28, 35). Although most of the ectoplasm and its extensions into the microvilli appeared to have been removed in the present study, the canaliculi still exhibited good morphologic integrity and resistance to disruption. The role of the ectoplasm, therefore, has probably been exaggerated, but the importance of the junctional complexes is obvious.

The low activity of glucose-6-phosphatase and the absence of cytochrome P-450 in our preparations of bile canaliculi (M-2) indicate only small, if any, contamination of these preparations with smooth-membrane vesicles derived from endoplasmic reticulum. The smooth vesicles present in M-2 (*S*, Fig. 4) must therefore originate largely from the noncanalicular portion of the plasma membrane as well as from some disrupted canaliculi.

Plasma membranes of the rat liver are known to contain only low concentrations of Na^+-K^+-activated ATPase (11, 12) in comparison to Mg^{++}-ATPase. Unlike other cell membranes such as erythrocyte ghosts (26) and microsomal preparations of the rat brain (5) in which the activity of this enzyme equals or exceeds that of the Mg^{++}-ATPase and in which an apparent activation of Mg^{++}-ATPase exceeding 100% can be brought about by Na^+ and K^+, hepatic plasma membranes in general show only approximately 30% activation (11), with occasional preparations showing lower or no activation at all (11). This makes the assay of Na^+-K^+-ATPase relatively difficult in hepatic plasma membranes and may account for, at least in part, our failure to observe Na^+-K^+-ATPase activity in about one-half of our preparations M-2.

The pH activity curves and the double pH optimum exhibited by 5'-nucleotidase in M-2 are similar to those observed in the rat (4, 33) and

human (32) livers and bull seminal plasma (19). The pronounced activation by Mg^{++} at pH values near 9.5 appears to be a characteristic of 5′-nucleotidases from those sources, and the kinetics of such activation have been discussed by Levin and Bodansky (19).

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