

Adenosine Mono-, Di- and Trinucleotidase Activities of Rat Liver Cytomembranes¹

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The liver continuously secretes triglycerides and cholesterol into the circulation in the form of very low density lipoproteins (6). The successful isolation of the various components of the secretory system (endoplasmic reticulum, Golgi apparatus and plasma membrane) from rat liver (7) provides new approaches to the identification and characterization of these structures. This report describes the procedure for the isolation of plasma membrane from rat liver adopted from those of Neville (8) and Emmelot et al. (4). The rates at which these plasma membrane fractions hydrolyze the mono-, di- and triphosphates of adenosine are compared with those of other components of the rat liver secretory system.

Materials and Methods

Isolation of plasma membrane. Male rats, 200 to 300 g (50 days old), purchased from the Holtzman Company, 421 Holtzman Road, Madison, Wisconsin, were anesthetized by intraperitoneal injections of 0.5 to 1 ml pentobarbital solution (Abbott Laboratories Nembutal, 20 mg/ml). Each liver was drained of blood, minced finely and homogenized in 5 to 10 ml 1 mM sodium bicarbonate using a very loose fitting Potter-Elvehjem all-glass tissue homogenizer (30 sec at 1,000 rpm). The homogenate was mixed with 300 ml of 1 mM sodium bicarbonate and stirred for 4 min, after which the suspension was filtered through a single layer of premoistened (with bicarbonate) cheesecloth followed by a second filtration through two layers of premoistened cheesecloth. The filtered homogenate was then centrifuged for 10 min at 3,500 rpm (about 1,500 *g*) using 50 ml centrifuge tubes and an angle rotor (Servall SS-34). The supernatant was discarded and the pellet was resuspended in 80 ml 1 mM bicarbonate. The 1,500 *g* centrifugation-resuspension cycles were continued until a clear supernatant was obtained (usually 3 to 4 times).

The final washed pellets were combined in 1 mM bicarbonate (total volume of 3 ml) and 9 ml of sucrose (density 1.3) was added slowly (by drops) with constant stirring. The following gradient was layered over the plasma membrane suspension: 5 ml sucrose density 1.20; 8 ml sucrose density 1.18; 5 ml sucrose density 1.16; 4 ml sucrose density 1.14. The gradient was then centrifuged for 75 min at 24,000 rpm (SW 25 rotor, Spinco Model L Ultracentrifuge). Plasma membrane was collected from the 1.16-1.18 density interface.

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Methods for obtaining Golgi apparatus and endoplasmic reticulum fractions will be described in detail elsewhere. These fractions were relatively free of plasma membrane and mitochondrial contamination (7).

Enzyme assays. Assays for 5'-mononucleodidase (EC 3.1.3.5) with AMP as substrate and ADPase (EC 3.6.1.6) were performed with the nucleotides (5 mM) as sodium salts in a medium containing 100 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris (pH 7.4) and a total volume of 2 ml with an incubation time of 15 min at 37°. For assays of the Na — K — Mg^{2+} —ATPase, the potassium salt of ATP was used in a medium

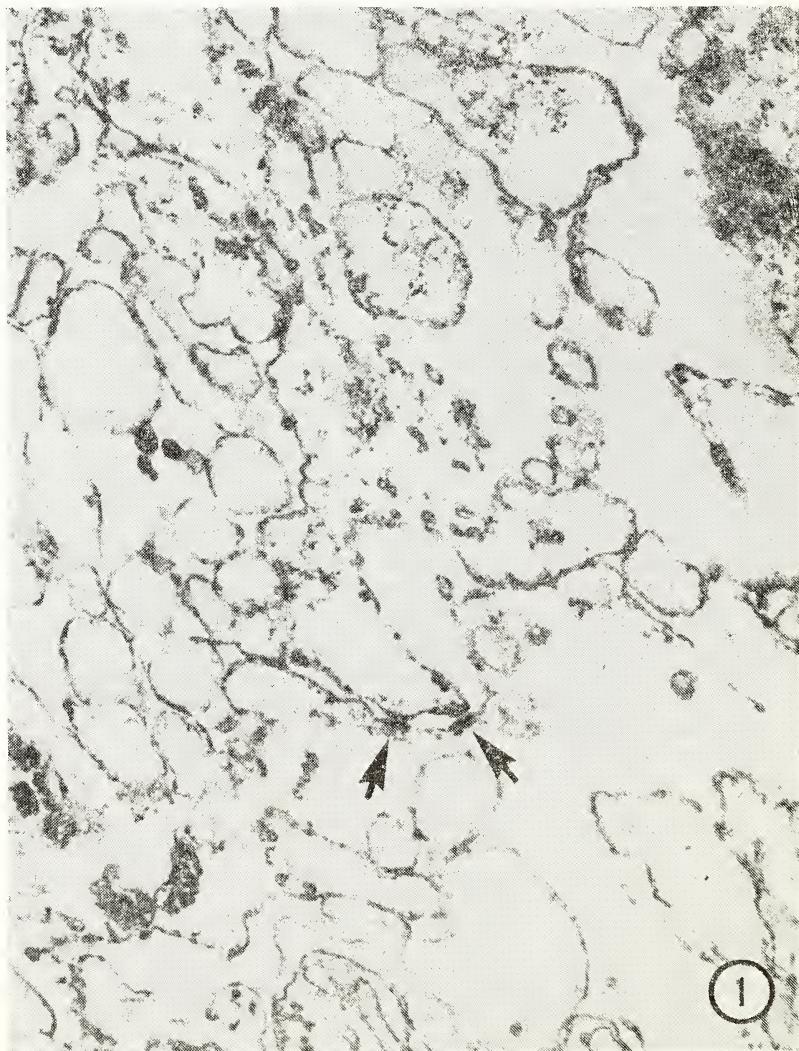


Figure 1. Thin section of plasma membrane pellet. Two junctional complexes are shown at arrows. Osmium fixation. X 33,300.

containing 66 mM NaCl, 33 mM KCl, 5 mM $MgCl_2$ and 25 mM Tris (pH 7.4). The corresponding medium for the Mg^{2+} —ATPase (EC 3.6.1.4) contained 100 mM KCl and no NaCl according to the procedure of Emmelot and Bos (3). Protein was determined by the Lowry procedure (5). The complete reaction mixtures contained approximately 0.1 mg protein.

Electron microscopy. Samples of isolated fractions were prepared for electron microscopy by fixation in 2% osmium tetroxide buffered to pH 7.1 with sodium phosphate (12 hrs, 4°) followed by dehydration and embedding in an Epon-Araldite plastic mixture. Negative staining of the unfixed plasma membrane fractions was done on carbon-stabilized, collodion-coated grids by first resuspending a portion of the pellet in water followed by mixing the suspension with an equal volume of 2% phosphotungstate (PTA) neutralized with potassium hydroxide to pH 6.8. Specimens were observed and photographed using a Philips EM/200.

Results and Discussion

Yield and purity of membranes. The yield of plasma membrane was 1 to 4 mg protein from 10 g fresh weight of liver. A 20-fold enrichment of 5'-nucleotidase and a 50-fold enrichment of Na^+ -activated ATPase relative to the total homogenate was obtained (Tables 1 and 2). The final pellets consisted of a pinkish-white layer of plasma membrane over a small layer of mitochondria. Based on estimates of succinic dehydrogenase (9), the activity of the plasma membrane fraction was 25% that of purified mitochondria. Glucose-6-phosphatase activity was 25% that of purified endoplasmic reticulum. Perhaps no more than 50% of the final pellet was plasma membrane.

Electron microscopy. Samples of preparations taken for electron microscopy contained vesicles of many sizes (Fig. 1). Occasionally a desmosome, a feature of the plasma membrane, was observed. Granules suggestive of ribosomes were not observed but mitochondria and mitochondrial fragments were prevalent.

TABLE 1. *Specific Activities of the ATPase and (Na^+ - K^+)—ATPase of the Plasma Membrane-Rich Cell Fraction and of the Total Homogenate from Rat Liver.*

Cell Fraction	Cations Added	Specific Activity (μ Moles iP/Hour/Mg Protein)		
		I	II	III
Total Homogenate	Mg^{++}, K^+	0.39	1.05	0.89
Plasma Membrane	Mg^{++}, K^+, Na^+	0.45	1.30	0.99
	ΔNa^+	+0.06	+0.25	+0.10
	Mg^{++}, K^+	2.9	15.2	10.8
	Mg^{++}, K^+, Na^+	5.0	23.2	19.6
	ΔNa^+	+2.1	+8.0	+8.8

TABLE 2. *Specific Activities of AMPase and ADPase of Rat Liver Cell Fractions.*

Cell Fraction	Specific Activity (μ Moles iP/Hour/Mg Protein)		
	AMP $\pm$ SD	ADP $\pm$ SD	ATP $\pm$ SD
Total Homogenate	1.2 $\pm$ 0.4	1.2 $\pm$ 0.3	0.7 $\pm$ 0.4
Plasma Membrane	23.0 $\pm$ 3.0	3.6 $\pm$ 2.6	9.6 $\pm$ 6.1
Golgi Apparatus	5.8 $\pm$ 0.3	1.7 $\pm$ 0.4	2.5 $\pm$ 0.9
Endoplasmic Reticulum	0.1 $\pm$ 0.8	0.1 $\pm$ 0.1	2.8 $\pm$ 1.3
Mitochondria	4.7 $\pm$ 3.1	0.9*	-----
Supernatant	1.2 $\pm$ 0.3	1.0*	0.65*

* Single determination

After staining with phosphotungstic acid (PTA), the collapsed membranes were embedded in a thin amorphous film of stain. Most of the membranes examined showed a fine granular structure in surface view with smooth edges. Occasionally, membrane margins exhibited globular knobs with an approximate diameter of 50 or 60 Å (Fig. 2). These knobs have been described as characteristic of certain regions of the plasma membrane (1, 2), probably the microvilli extruding into the bile spaces (Benedetti and Emmelot, unpublished). In addition, a hexagonal array of subunits was observed in certain membrane sheets (Fig. 3) as previously reported by Benedetti and Emmelot (1). The hexagonal subunit patterns were prevalent in preparations containing tight junctions (2).

Sodium-potassium activated ATPase. The specific activities of the Mg^{2+} -ATPase and $(Na^{+}-K^{+})$ -ATPase of freshly prepared plasma membrane (Table 1) showed considerable variation from preparation to preparation. This is consistent with reports for other membrane ATPase systems (3). The $(Na^{+}-K^{+})$ -ATPase is the Na^{+} -mediated increase of inorganic phosphate released from ATP over that observed in the absence of Na^{+} with adjustment of $[K^{+}]$ to maintain osmolarity (Table 1). Sodium ions increased the inorganic phosphate released from ATP in the presence of potassium ions by most plasma membrane preparations to about 170% that obtained in the absence of sodium ions. Results with Golgi apparatus and endoplasmic reticulum fractions were variable and inconclusive.

5'-Nucleotidase. As summarized in Table 2, the 5'-nucleotidase (AMPase) activities of the plasma membrane fractions were significantly higher than either endoplasmic reticulum or Golgi apparatus. This activity appears to be localized as an intrinsic constituent of the liver plasma membrane and serves as a convenient measure of plasma membrane contamination of these cell fractions.

ADPase. In contrast to previous reports of Emmelot and Bos (3), plasma membrane fractions obtained by our procedures showed a low level of ADPase activity (Table 2). It has not been verified that this increase in inorganic phosphorus is due to a genuine ADPase.

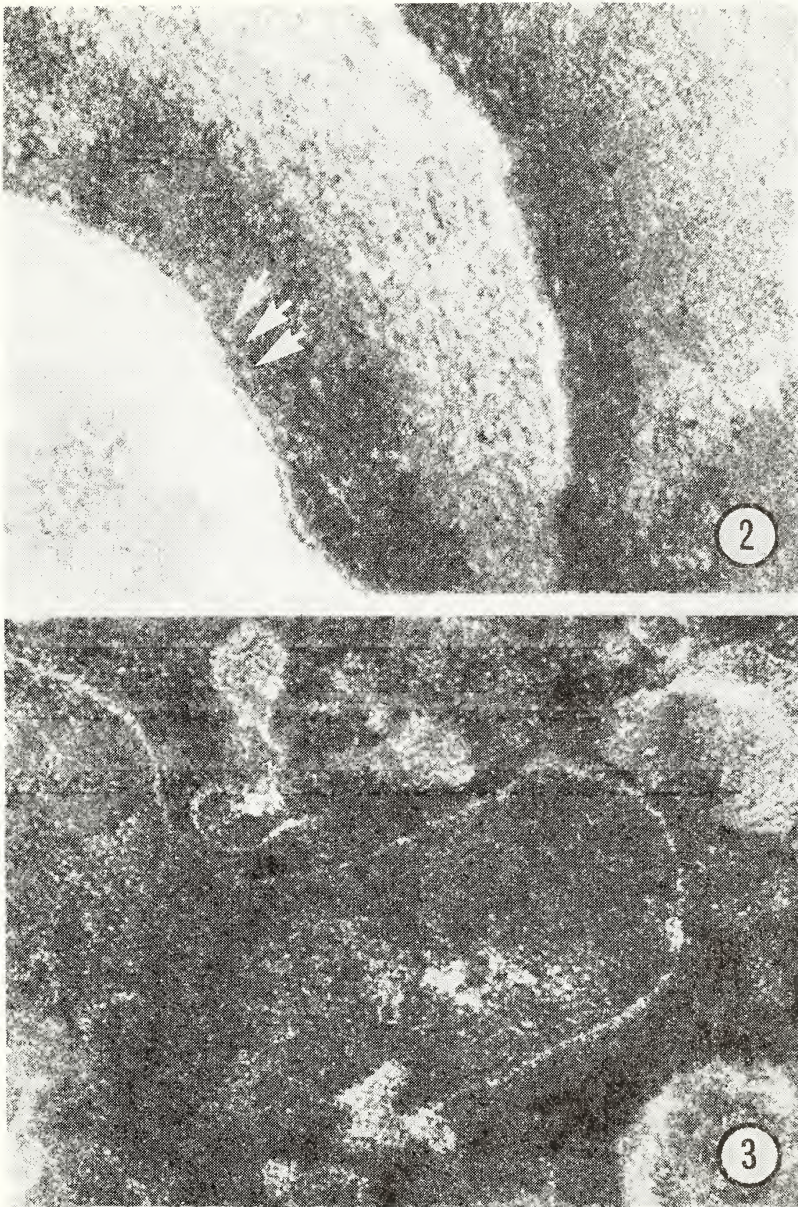


Figure 2. Liver plasma membrane negatively stained with phosphotungstic acid. Globular knobs are found at the edge of some membrane sheets (arrows). X 300,000.

Figure 3. Liver plasma membrane negatively stained with phosphotungstic acid. The surface is dotted with small particles in hexagonal array. X 90,000.

Summary

A plasma membrane-rich cell fraction isolated from rat liver was shown to contain both a Mg^{2+} —ATPase and a sodium-potassium-activated ATPase. The fraction was characterized by a high 5'-nucleotidase activity with AMP as substrate. This activity was low or absent in isolated Golgi apparatus and endoplasmic reticulum fractions. The absence of AMPase in other cytomembrane fractions and the ease and unambiguity of its estimation makes AMPase a useful marker enzyme for plasma membrane.

Literature Cited

1. BENEDETTI, E. L., and P. EMMELLOT. 1965. Electron microscopic observations on negatively stained plasma membranes isolated from rat liver. *J. Cell Biol.* **26**:299-305.
2. BENEDETTI, E. L., and P. EMMELLOT. 1968. Hexagonal array of subunits in tight junctions separated from isolated rat liver plasma membranes. *J. Cell Biol.* **38**:15-24.
3. EMMELLOT, P., and C. J. BOS. 1966. Studies on plasma membranes. III. Mg^{2+} — ATPase, $(Na^{+}-K^{+}-Mg^{2+})$ —ATPase and 5'-nucleotidase activity of plasma membranes isolated from rat liver. *Biochim. Biophys. Acta* **120**:369-382.
4. EMMELLOT, P., C. J. BOS, E. L. BENEDETTI, and P. RUMKE. 1964. Studies on plasma membranes. I. Chemical composition and enzyme content of plasma membranes isolated from rat liver. *Biochim. Biophys. Acta* **90**:126-145.
5. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
6. MIDDLETON, A. E., D. J. MORRE', L. M. ALVES, R. L. HAMILTON, and R. MAHLEY. 1968. Immunochemical identification of very low density serum lipoproteins in Golgi apparatus from rat liver. *Proc. Indiana Acad. Sci.* **77**:159-163.
7. MORRE', D. J., R. CHEETHAM, and W. YUNGHANS. 1968. Biochemical characterization of a Golgi apparatus-rich cell fraction isolated from rat liver. *J. Cell Biol.* **39**:96a.
8. NEVILLE, D. M. 1960. The isolation of a cell membrane fraction from rat liver. *J. Biophys. Biochem. Cytol.* **8**:413-422.
9. PENNINGTON, R. J. 1961. Biochemistry of dystrophic muscle. Mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. *Biochem. J.* **80**:649-654.