

# HELa CELL PLASMA MEMBRANES

## I. 5'-Nucleotidase and Ouabain-Sensitive ATPase as Markers for Plasma Membranes

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### ABSTRACT

A method for the preparation of HeLa cell plasma membrane ghosts is described. The purity of the plasma membrane fraction was examined by phase contrast and electron microscopy, by chemical analysis, and by assay of marker enzymes. Data on the composition of the plasma membrane fraction are given. It was observed that the distribution pattern of 5'-nucleotidase activity among the subcellular fractions differed from that of ouabain-sensitive ATPase. In addition, the specific activity of 5'-nucleotidase did not follow the distribution of the membrane ghosts. Thus, this enzyme would seem unsuitable as a plasma membrane marker. A complete balance sheet for marker enzyme activities during the fractionation is necessary for the calculation of increase in specific activity because the activities of both 5'-nucleotidase and ouabain-sensitive ATPase might change during the fractionation procedures.

The purification of plasma membranes is usually evaluated by the activity of marker enzymes, especially 5'-nucleotidase and ouabain-sensitive ATPase (1). 5'-Nucleotidase was shown by Essner et al. (2) to be related to the plasma membrane in rat hepatocytes and has subsequently been used in the estimation of the purity of plasma membranes isolated from several other cell types including cells in tissue culture (3-5).

We report a novel method for the purification of HeLa cell plasma membranes and demonstrate that the distribution of the activity of 5'-nucleotidase over the fractions differs from that of ouabain-sensitive ATPase. It seems that 5'-nucleotidase activity is not limited to the plasma membrane in HeLa cells. We also show that the presence of EDTA during the isolation of plasma membranes constitutes a source of error in the evaluation of the purification obtained.

### MATERIALS AND METHODS

#### *Culture Methods*

Suspensions of HeLa S<sub>3</sub> cells were grown in 1,000-ml Florence flasks in antibiotic-free Eagle's minimum essential spinner medium supplemented with 0.1 mM glycine, 0.1 mM serine, and 10% calf serum.

#### *Isolation of Plasma Membranes*

Log-phase cells (about  $3-4 \times 10^6$ /ml) were harvested and washed twice in saline buffered with 1 mM Ca acetate, pH 7.0. The cells were then suspended ( $5 \times 10^7$  cells/ml) in 10 mM Tris buffer, pH 7.0, allowed to swell for 10 min, and gently homogenized in a tight-fitting Dounce homogenizer. The process was controlled by phase microscopy and stopped when most of the cells were ruptured and many whole membranes were seen. About 20 strokes proved optimal.

The nuclei were stabilized by adding 1 vol 20% wt/wt sucrose in 10 mM Tris-HCl buffer, pH 7.0, and removed

by centrifugation at an average  $g$ -min value of 680 (2,000 rpm/1 min) at room temperature in 30-ml Correx tubes with the HL-4 rotor in a Sorvall GLC-1 centrifuge (Ivan Sorvall, Inc., Newtown, Conn.). This procedure removed 90% of the nuclei as evidenced by DNA determination. Centrifuging the supernate at 8,000  $g$ -min (1,600 rpm/20 min) in 30-ml Correx tubes at 2°C in the HB-4 rotor of the Sorvall IRC-2B centrifuge sedimented the whole plasma membranes, whereas most of the other organelles remained in the supernate. The pellet, constituting a crude plasma membrane fraction, was suspended in 30% wt/wt sucrose in 10 mM Tris buffer, pH 7.0, layered on a continuous 30–50% wt/wt sucrose gradient, and submitted to isopycnic centrifugation at 50,000  $g$  for 15 h in the Spinco SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C. This resulted in a sharp band of plasma membranes. The band was removed. After being washed three times, the plasma membranes were suspended in 10 mM Tris buffer, pH 7.0. In some experiments 5 mM EDTA was included in all buffers and washing fluids.

### Chemical Analysis

The resulting plasma membrane fraction was analyzed for protein, lipids, RNA, DNA, and carbohydrates. Protein was also determined in the other fractions isolated. Protein was precipitated by an equal volume of 2% phosphotungstic acid in 1 N HCl, dissolved in 1 N NaOH, and measured according to Lowry et al. (6) with bovine serum albumin as standard.

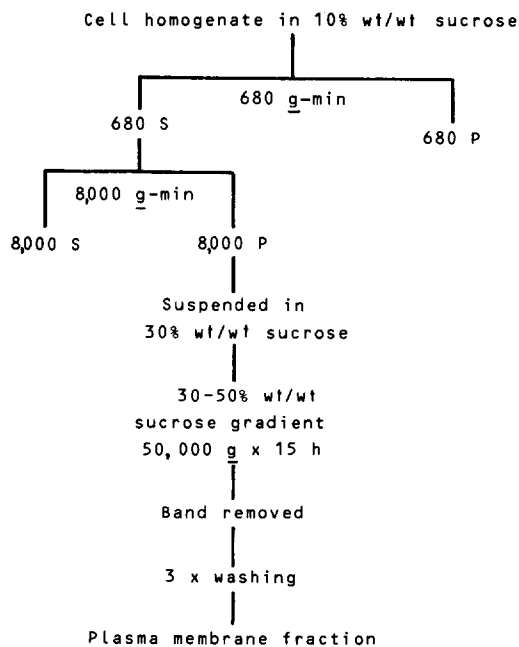


FIGURE 1 Flow chart for the purification of plasma membranes from HeLa cells. P, pellet; S, supernate.

Lipids were extracted from the whole homogenate and from plasma membrane suspensions by adding 9 vol chloroform-methanol (2:1 vol/vol) to 1 vol of aqueous suspension, or alternatively, directly from plasma membrane pellets. All extractions were carried out at 37°C for 1 h and repeated once.

Cholesterol was measured by the Liebermann-Burchard reaction (7). Phospholipids were hydrolyzed in 50  $\mu$ l  $H_2SO_4$  and 0.5 ml  $HNO_3$ , and inorganic phosphorus was determined according to Chen et al. (8). The phospholipid content was calculated by assuming a phosphorus content of 4%.

RNA and DNA were precipitated in 0.5 M cold perchloric acid (PCA), and the precipitate was washed twice with 0.5 M PCA. RNA was hydrolyzed in 0.3 M KOH at 37°C for 16 h and determined by the orcinol reaction (9), with ribose as standard. DNA was hydrolyzed in 0.5 M PCA at 90°C for 20 min and determined according to Burton (10), with 2-deoxyribose as standard.

Sialic acids were liberated by hydrolysis in 0.05 M  $H_2SO_4$  at 80°C for 1 h and estimated by the thiobarbituric acid method (11), correction for interfering substances being made by reading the optical densities at 549 nm and 532 nm (11). Fucose, hexosamine, and galactose were determined after hydrolysis in 3 N HCl at 100°C for 5 h, fucose by the method of Gibbons (12), hexosamine by the method of Johnson (13), and D-galactose with the galactose oxidase kit (Galax) from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

### Assay of Marker Enzymes

In each fraction the activities of 5'-nucleotidase (EC 3.1.3.5), ouabain-sensitive ( $Na^+$ ,  $K^+$ )-ATPase (EC 3.6.1.3), NADPH-cytochrome reductase (EC 1.6.2.4), cytochrome *c* oxidase (EC 1.9.3.1),  $\beta$ -*N*-acetylglucosaminidase (EC 3.2.1.30), NAD(P)H dehydrogenase (DT-diaphorase) (EC 1.6.99.2), and the protein content were measured. In addition, the activities of cytochrome *c* oxidase and  $\beta$ -*N*-acetylglucosaminidase were determined in a crude mitochondrial fraction isolated according to Attardi et al. (14).

5'-Nucleotidase activity was determined (15) by incubation of 0.1 ml homogenate with 0.9 ml of a solution containing 6 mM 5'-AMP, 0.1 M glycine buffer, pH 8.5, and 10 mM  $MgCl_2$  for 30 min at 37°C. The reaction was stopped with 1.0 ml cold 10% trichloroacetic acid, and the precipitate separated by centrifugation. Free phosphate was measured according to Chen et al. (8). To exclude unspecific phosphatase activity, the corresponding activity against 2'(3')-AMP under the same conditions was subtracted.

Ouabain-sensitive ( $Na^+$ ,  $K^+$ )-ATPase was determined (16) by incubation of 0.1 ml homogenate with 0.9 ml substrate solution containing 2 mM ATP, 0.04 M histidine-imidazole buffer, pH 7.1, 80 mM NaCl, 33 mM KCl, and 2 mM  $MgCl_2$  for 60 min at 37°C. The final

ouabain concentration was 1 mM. The reaction was stopped and free phosphate measured as above.

The activity of NADPH cytochrome reductase was determined according to Ernster (17), cytochrome *c* oxidase according to de Duve et al. (18),  $\beta$ -*N*-acetylglucosaminidase according to Barrett (19), and DT-diphosphorase according to Scott and McGraw (20).

### Electron Microscopy

Plasma membranes were fixed in glutaraldehyde- $H_2O_2$ , postfixed in osmium tetroxide, and stained by uranyl acetate as described by Peracchia and Mittler (21). The specimens were dehydrated in ethanol and propylene oxide and embedded in Epon. Thin sections were cut on an LKB Ultratome III (LKB Instruments, Inc., Rockville, Md.), poststained in 3% lead citrate (22), and examined with a Hitachi HU-12 electron microscope.

## RESULTS

### Composition of Fractions

Most of the plasma membrane ghosts present in the homogenate remained in the supernate after centrifugation for 680 *g*-min in 10% wt/wt sucrose. In the pellet nuclei, unruptured cells and some membranes were found.

When the supernate was centrifuged 8,000 *g*-min, almost all of the ghosts were pelleted together with some remaining nuclei and cells. The 8,000 *g*-min supernate contained endoplasmic reticulum fragments, ruptured plasma membranes, and cytoplasmic organelles.

During the isopycnic centrifugation in the linear sucrose gradient, the plasma membranes were separated from the rest of the nuclei and whole cells. The yield of plasma membranes was 15–20% as calculated from the recovery of ouabain-sensitive ATPase and on the assumption that the enzyme is a true plasma membrane marker.

### Physical Characteristics of Plasma Membranes

Under phase-contrast microscopy the plasma membranes appeared as whole ghosts free from contaminants (Fig. 2). The electron micrographs showed large bilayer membrane sheets and very few other organelles except for some particles of about 150-Å diameter, which were probably ribosomes (Fig. 3), and some amorphous material probably of protein nature. In the sucrose gradient the membranes banded at 1.20 *g*/ml, but in the

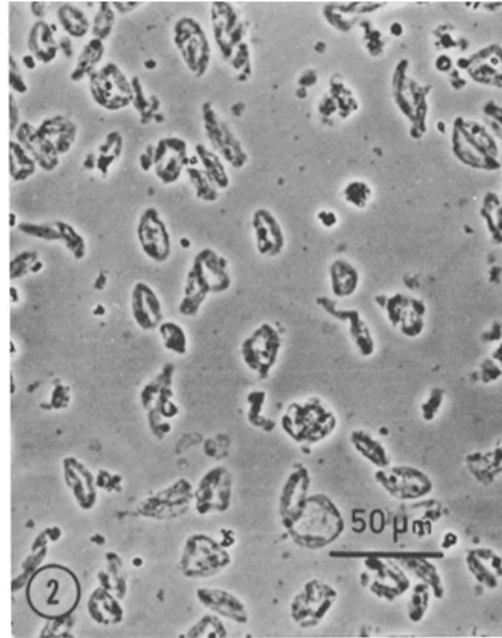


FIGURE 2 Phase-contrast micrograph of isolated plasma membrane ghosts.  $\times 320$ .

presence of EDTA the density changed to 1.16 *g*/ml.

### Chemical Composition of Plasma Membranes

The relative composition of the plasma membrane fraction expressed per milligram protein is shown in Table I. An experiment based on  $10^9$  cells, i.e. about 250 mg protein, showed approximately 4 mg protein in the purified plasma membranes. The RNA content of the membranes was essentially unchanged when EDTA was present during the isolation.

### Marker Enzymes

The distribution of protein, ouabain-sensitive ( $Na^+$ ,  $K^+$ )-ATPase, 5'-nucleotidase, cytochrome *c* oxidase, NADPH-cytochrome reductase, DT-diphosphorase, and  $\beta$ -*N*-acetylglucosaminidase is shown in table II.

Ouabain-sensitive ATPase activity was found in all fractions, indicating some loss of plasma membranes during the isolation procedure, either as whole cells in the 680 *g* pellet or as small fragments in the 8,000 *g* supernate (Table II). The pattern of 5'-nucleotidase was different, with a relatively

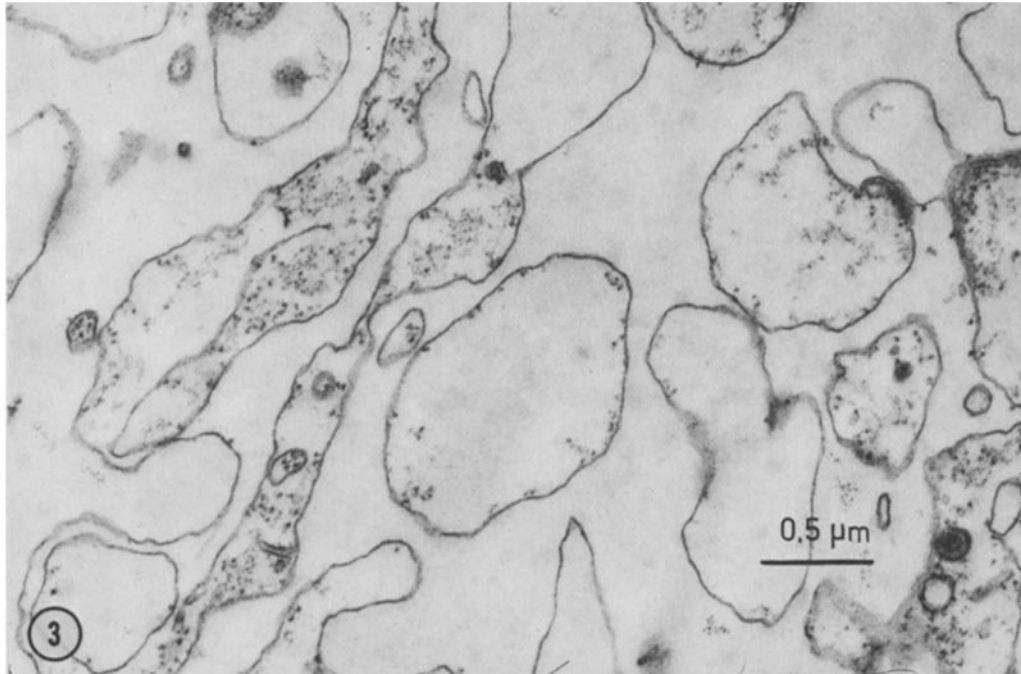


FIGURE 3 Electron micrograph of plasma membrane fraction.  $\times 20,000$ .

TABLE I  
*Chemical Composition of Plasma Membranes*

	Content
	$\mu\text{g}/\text{mg protein}$
Phospholipid	464
Cholesterol	183
Sialic acid	8.1
Fucose	3.0
Hexosamine	14.0
Galactose	4.5
RNA	22.0
DNA	Not detected ( $< 1.9$ )

higher content in the 8,000 g supernate and a lower content in the 8,000 g pellet. This difference between the two enzymes reappeared when the specific activities were calculated for the various fractions, and was further enlarged in the purified plasma membrane fraction giving a purification of 19-fold for ouabain-sensitive ATPase and 7-fold for 5'-nucleotidase.

Cytochrome *c* oxidase, NADPH-cytochrome reductase, DT-diaphorase, and  $\beta$ -*N*-acetylglucosaminidase activities were found mostly in the 8,000 g supernate (Table II). However, some activity of

these enzymes was found in the plasma membrane fraction, indicating contamination by mitochondria, endoplasmic reticulum, and lysosomes. These contaminants were not removed by repeated washings of the plasma membranes and were presumably attached to the inside of the ghosts. The plasma membrane/mitochondria ratio, as indicated by the specific activities of their marker enzymes, was 10-fold higher in the final plasma membrane preparation than in the whole homogenate. Similarly, the plasma membrane/lysosome ratio and the plasma membrane/endoplasmic reticulum ratio were 20-fold higher.

A mitochondrial fraction isolated by sucrose gradient centrifugation had a specific activity of 8.0 nkatals/mg for cytochrome *c* oxidase and 1.02 nkatals/mg for  $\beta$ -*N*-acetylglucosaminidase.

The distribution of 5'-nucleotidase and ouabain-sensitive ATPase was compared with and without EDTA in the buffer solutions. The presence of EDTA reduced the 5'-nucleotidase activity by 40–50% in each purification step, giving a very low final recovery. On the other hand, in the absence of EDTA, the ouabain-sensitive ATPase activity was lowered, especially in the whole cell homogenate, giving anomalously high recoveries (140–150%).

The EDTA effect remained if the Ca acetate buffer in the washing fluids was replaced by a Tris-HCl buffer. The inactivation of 5'-nucleotidase by EDTA was followed in a cell homogenate stored at 5°C. The activity decreased to 5% after 24 h as shown in Fig. 4. Adding an excess of Mg<sup>2+</sup> or freezing at -20°C prevented the inactivation, but lost activity could not be restored by Mg<sup>2+</sup>.

## DISCUSSION

The procedure of the plasma membrane isolation is a modification of the methods described by Bosmann et al. (5) and Atkinson and Summers (23). The gentle homogenization preserved the plasma membranes as ghosts, which may be subsequently isolated by a combination of velocity and isopycnic centrifugations. No cross-linking

agent of the type used by Warren et al. (24) was found necessary to obtain whole ghosts from HeLa cells grown in suspension. With our method the purification process can be easily monitored by morphological methods and the membranes are at the same time intact for enzymatic and other biochemical studies. On the other hand, plasma membranes of HeLa cells trypsinized from surface cultures are more fragile and will not produce whole ghosts under the same conditions.

During the homogenization some membrane ghosts are disintegrated while other cells are left unbroken. In both cases plasma membrane material is lost. Some ghosts also disintegrate during the fractionation. Thus, the yield of 15-20% in the final plasma membrane fraction is quite reasonable. The protein recovered in this fraction consti-

TABLE II  
*Distribution of Protein and Marker Enzymes*

	H	680 S	680 P	8,000 S	8,000 P	M
Protein						
%	100	70	27	58	11	1
Ouabain-sensitive ATPase						
%	100	81	30	35	48	21
Sp act	0.19*	0.28	0.11	0.17	0.83	3.75
5'-Nucleotidase						
%	100	67	23	38	24	6
Sp act	0.61	0.92	0.22	0.70	1.72	4.28
Cytochrome c oxidase						
%	100	62	22	64	7	2
Sp act	0.71	0.77	0.49	0.93	0.61	1.07
NADPH-cytochrome reductase						
%	100	65	11	65	8	1
Sp act	0.34	0.39	0.12	0.45	0.30	0.23
DT-diaphorase						
%	100	92	5	85	2	N.D.
Sp act	9.7	13.8	1.8	15.5	2.0	<1.8
$\beta$ -N-acetylglucosaminidase						
%	100	67	32	58	10	1
Sp act	0.86	1.00	0.86	1.02	0.94	0.68

H, whole homogenate; 680 S, 680 g-min supernate; 680 P, 680 g-min pellet; 8,000 S, 8,000 g-min supernate; 8,000 P, 8,000 g-min pellet; M, plasma membrane fraction; N.D., not detected.

\* The specific activities are given in nanokatal per milligram.

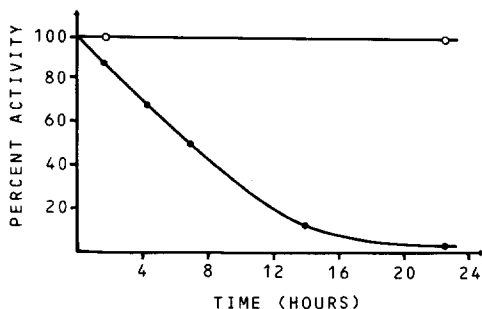


FIGURE 4 Whole cell homogenate incubated at 5°C in 5 mM Tris buffer, pH 7.0, with (●—●) or without (○—○) 5 mM EDTA. Final concentrations in the enzyme assay were EDTA 0.5 mM,  $Mg^{2+}$  11 mM.

tutes about 1% of total protein in the whole homogenate, i.e., the plasma membrane contains 5–6% of total cell protein. Slightly higher values (1–2%) were recovered when a large number of cells (i.e., above  $10^9$ ) were used.

The electron micrographs show that the plasma membranes are quite well preserved as ghosts, the only contaminants present being ribosomes and some amorphous material, probably of protein nature. Very few mitochondria and lysosomes were seen. The chemical composition as given in Table I is in good agreement with earlier determinations on plasma membranes from HeLa cells (5). A high molecular ratio of cholesterol/phospholipid has been shown to be a characteristic of plasma membranes in various cell types. In the plasma membrane fraction we find a ratio of 0.81, which is 3.5 times higher than the ratio in the whole cell homogenate, but somewhat lower than earlier reported (5) for HeLa cells.

The RNA content of 22  $\mu$ g per mg protein is presumably due to a ribosomal contamination as revealed by electron microscopy. The nature of the RNA is under study. The microsomal and cytoplasmic marker enzymes indicated a contamination by endoplasmic reticulum of less than 5% when compared with whole homogenate. If the maximum ratio of RNA/protein in rough endoplasmic reticulum in HeLa cells is the same as that of liver (0.19) (25), more than 10% microsomal contamination is necessary to explain the RNA present. Furthermore, the presence of EDTA during membrane isolation did not reduce the RNA content of the final plasma membrane preparations. A reduction might have been expected due to release of ribosomal subunits if ribosomal RNA bound to endoplasmic reticulum were present. The ribosomes are therefore most

probably not present as rough endoplasmic reticulum, i.e., bound to internal membranes. They are either trapped within the membrane ghosts or bound to the plasma membrane itself in a way different from the way in which they are bound to the endoplasmic reticulum. A close inspection of the electron micrographs revealed no evidence for the second alternative. 5'-Nucleotidase has been generally accepted as a marker enzyme of plasma membranes in several cell types including HeLa cells, and the enzyme has been used to estimate the purity of plasma membrane preparations.

Our results, based on quantitative recovery and analysis of all fractions, demonstrate that 5'-nucleotidase does not copurify with ouabain-sensitive ATPase. The isolation procedure allows us to use morphological criteria besides the marker enzymes, and this reveals that the activity of ouabain-sensitive ATPase follows strictly the plasma membranes while a proportionally greater part of the 5'-nucleotidase activity is found in the 8,000-g supernate. This suggests that 5'-nucleotidase in HeLa cells is not uniquely located in the plasma membrane and is therefore unsuitable as a marker enzyme.

When marker enzymes are used for quantitative estimates of purification of various subcellular fractions, it is necessary to ascertain that the markers are not inhibited or stimulated during the fractionation. EDTA is very often used in the fractionation of plasma membranes, since  $Ca^{++}$  inhibits the membrane ATPase (26). We show that EDTA inactivates 5'-nucleotidase (Fig. 4).

The 120-fold purification of 5'-nucleotidase in a HeLa cell plasma membrane fraction reported earlier (5) could well be explained by the inclusion of EDTA in the reference homogenate and its absence in the membrane fraction, thereby giving too low a reference activity and consequently too high a specific activity for the membrane fraction.

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