ISOLATION OF PLASMA MEMBRANE FRAGMENTS FROM HELA CELLS

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ABSTRACT

A method for isolating plasma membrane fragments from HeLa cells is described. The procedure starts with the preparation of cell membrane "ghosts," obtained by gentle rupture of hypotonically swollen cells, evacuation of most of the cell contents by repeated washing, and isolation of the ghosts on a discontinuous sucrose density gradient. The ghosts are then treated by minimal sonication (5 sec) at pH 8.6, which causes the ghost membranes to pinch off into small vesicles but leaves any remaining larger intracellular particulates intact and separable by differential centrifugation. The ghost membrane vesicles are then subjected to isopycnic centrifugation on a 20–50% w/w continuous sucrose gradient in tris-magnesium buffer, pH 8.6. A band of morphologically homogeneous smooth vesicles, derived principally from plasma membrane, is recovered at 30–33% (peak density = 1.137). The plasma membrane fraction contained a Na-K-activated ATPase activity of 1.5 μmole Pi/hr per mg, 3% RNA, and 13.8% of the NADH-cytochrome *c* reductase activity of a heavier fraction from the same gradient which contained mitochondria and rough endoplasmic vesicles. The plasma membranes of viable HeLa cells were marked with ^{125}I-labeled horse antibody and followed through the isolation procedure. The specific antibody binding of the plasma membrane vesicle fraction was increased 49-fold over that of the original whole cells.

INTRODUCTION

The architectural organization of cells within tissues appears to be maintained, at least in part, through a system of contact-dependent signals between the cells (21). The development of specific tissue configurations during embryogenesis may also depend on such a system (3, 6, 15). Certain properties of cultured fibroblasts, which bear on their spatial orientation and growth in tissues, have been shown to depend on the contact-mediated transmission of information between cells. These are contact inhibition of locomotion (1), of mitosis (16), and of nucleic acid and polysome synthesis (9). Since a major defining tissue characteristic of neoplasia is a disturbance of the normal spatial orientation of cells, it is reasonable to consider that a permanent breakdown in contact-mediated cell communications may be the immediate basis for the neoplastic process. Because one possible mechanism to account for this breakdown is a structural change at the site of cell contact, i.e. in the plasma membrane, we are currently engaged in determining structural differences between the plasma membranes of normal and neoplastic cells. The first phase of this work, the development of a method for isolating the plasma membranes of mammalian cells, is reported below.

MATERIALS AND METHODS

Cells and Solutions

A standard tris-magnesium (TM) buffer was used at either pH 7.4 or pH 8.6: 0.01 M Tris, 0.001 M $MgCl_2$. 100 × TM stock solutions were prepared and adjusted with concentrated HCl to give a pH of either 7.4 or 8.6 at 4°C after dilution. Other buffered solutions were prepared from the 100 × stock as follows: TM saline, by diluting TM stock with 0.85% NaCl; TM-sucrose, by diluting the appropriate TM stock with 0.25 M sucrose.

Suspension culture strain S3 of HeLa cells, recently cloned, was grown at 37°C in multiple 6-liter flasks, with Eagle's MEM,[1] Earle's salts without calcium and with 10 times the usual amount of phosphate, 5% fetal calf serum, and 2 mM glutamine. Cells were harvested aseptically by centrifuging the culture fluid in 500 ml bottles at 1,000 rpm for 10 min, 4°C, in the IEC Model PR-2 centrifuge, diluted with medium to a cell count of 6.24×10^7/ml, mixed with an equal volume of cold 15% dimethylsulfoxide in complete medium, viable-frozen in a Linde BF-3 apparatus (Linde Division, Union Carbide Corp., N. Y.) at a cooling rate of 1°C per min, and stored in 16-ml aliquots in screw-cap vials over liquid nitrogen. Before a plasma membrane isolation procedure was begun, the cells were rapidly thawed in water at 37°C, and washed three times with 10 volumes of ice-cold TM-saline, pH 7.4, by suspension and centrifugation at 1,000 rpm for 5 min, 4°C. Cells that showed less than 95% viability by the Trypan blue dye exclusion method (final dye concentration 0.17%) after they were washed were not used. An average of 10 ml of packed cells (equivalent to approximately 7 vials, 3.5×10^9 cells, or 3.5 liters of original suspension culture) was used in a typical plasma membrane isolation experiment.

Plasma Membrane Isolation Procedure

The final protocol for isolating plasma membranes is itemized below. Comments and findings pertaining to the different steps are given in the Results and Discussion sections. All procedures are carried out in crushed ice at 0°C.

STEP 1. HYPOTONIC TREATMENT AND CELL RUPTURE: Approximately 3.5×10^9 cells (10 ml packed cells) are suspended in 100 ml of ice-cold TM, pH 7.4. Cell swelling is monitored by phase-contrast microscopy, and reaches a maximum in less than 5 min. When this occurs, the suspension is placed in a 50 ml Dounce homogenizer fitted with a tight (Type B) pestle (Bellco Glass Inc., Vineland, N.J.), and is subjected to 3–5 downstrokes of the pestle (5 sec per downstroke, air pocket avoided on return stroke). The degree of cell rupture is checked by phase-contrast microscopy after each stroke. 95% cell rupture is sufficient. 60% (w/w) sucrose is added immediately to make a final concentration of 0.25 M sucrose (the cell membrane ghosts, described below, break apart within a few minutes in TM and TM-saline but are stable for 24 hr or longer in sucrose solutions at concentrations of 0.25 M or higher). The suspension of ruptured cells is centrifuged at 3,000 rpm (rotor No. 269, IEC Model PR-2) for 15 min, 4°C, in 50-ml graduated, conical polycarbonate centrifuge tubes. It is important to use plastic tubes because the ghosts adhere strongly to glass. A laminated pellet is produced in which the topmost lighter gray lamina consists almost entirely of cell ghosts. The entire pellet is processed, however, because this gives a higher final yield. The pellet is washed three times with 20 volumes of TM-sucrose, pH 7.4, by repeated suspension and centrifugation at 3,000 rpm for 15 min, 4°C.

STEP 2. SEPARATION OF THE GHOST FRACTION BY FLOTATION IN A DISCONTINUOUS SUCROSE DENSITY GRADIENT: A small excess of supernatant is left over the washed pellet, which now contains ghosts, nuclei, and incompletely ruptured whole cells. Enough 60% w/w sucrose in TM, pH 8.6, is added to give a reading of 45% sucrose on a refractometer (Bausch & Lomb, Incorporated, Rochester, N.Y.). After this step, all solutions contain TM, pH 8.6. 15 ml of the suspension are placed in each of three 50 ml SW-25.2 cellulose nitrate tubes, and discontinuous gradients are formed over the suspensions by adding 15-ml layers, respectively, of 40% and 35% w/w sucrose, and then adding 5 ml of TM-sucrose (0.25 M). The material is now centrifuged at 20,000 rpm for 10 min, 4°C, in the SW-25.2 rotor. The nuclei sediment and form a pellet, the incompletely ruptured whole cells collect at the 40%/45% interface, and the ghosts collect at the 35%/40% interface. The ghost bands are collected with a pipette and bulb and are pooled. The pool is diluted with TM, pH 8.6, to give a refractometer reading of 10% w/w sucrose, and centrifuged at 3,500 rpm for 20 min, 4°C, in polycarbonate centrifuge tubes. The pellet of ghosts is then resuspended in 6 ml of TM-sucrose, pH 8.6.

STEP 3. SONICATION OF GHOSTS FOLLOWED BY LOW-SPEED CENTRIFUGATION: The suspension of ghosts in the plastic centrifuge tube is now sonicated for exactly 5 sec, by using an MSE sonicator with microprobe, at an amplitude setting of 8 (Instrumentation Associates, N.Y.). This short period of sonication is enough to cause the plasma membranes of the ghosts to break up into microvesicles, but is not sufficient to disrupt the few mitochondria and other structures which have

[1] Abbreviations: MEM, minimum essential medium; IEC, International Equipment Company.

remain trapped within the ghosts. Much of this formerly trapped material is then removed from the suspension by centrifugation at 3,000 rpm for 15 min, 4°C. The resulting "sonicated ghost supernatant" is saved.

STEP 4. PURIFICATION OF PLASMA MEMBRANE VESICLES BY SEDIMENTATION IN A CONTINUOUS SUCROSE DENSITY GRADIENT: The sonicated ghost supernatant is top-loaded on a continuous 20–50% w/w sucrose density gradient containing TM, pH 8.6, and centrifuged in the SW-25.1 rotor at 20,000 rpm for 16 hr at 4°C. The gradient is then top-unloaded in 0.5–1.0 ml fractions by using a Beckman gradient tapping assembly (SW-25.1 Fractionation System, Spinco Div., Beckman Instruments, Inc. Palo Alto, Calif.) and a Sage syringe pump (Model 255-1, Sage Instruments, Inc., White Plains, N.Y.). The optical density of the effluent fractions is monitored with a spectrophotometer flow cell and strip chart recorder at a wavelength setting of 280 mμ. The material in the various fractions is diluted with TM, pH 8.6, to a sucrose concentration of 10% or less and pelleted in the Spinco No. 40 fixed angle rotor at 40,000 rpm for 45 min, 4°C. Spinco 10-ml cellulose nitrate tubes with aluminum caps are used. The pellets are then ready for electron microscopic examination, radioisotope counting, enzyme assay, etc.

Biochemical Assays

Magnesium-dependent, sodium- and potassium-stimulated, ouabain-sensitive ATPase was determined by the method of Wallach and Ullrey (19). Assays were done in duplicate, by using at least 70 μg of sample protein per tube and incubating at pH 8.4 for 1 hr.

NADH-cytochrome *c* reductase was determined by the method of Mahler (11), ribonucleic acid by the method of Fleck and Munro (5), and protein by the method of Lowry et al. (10).

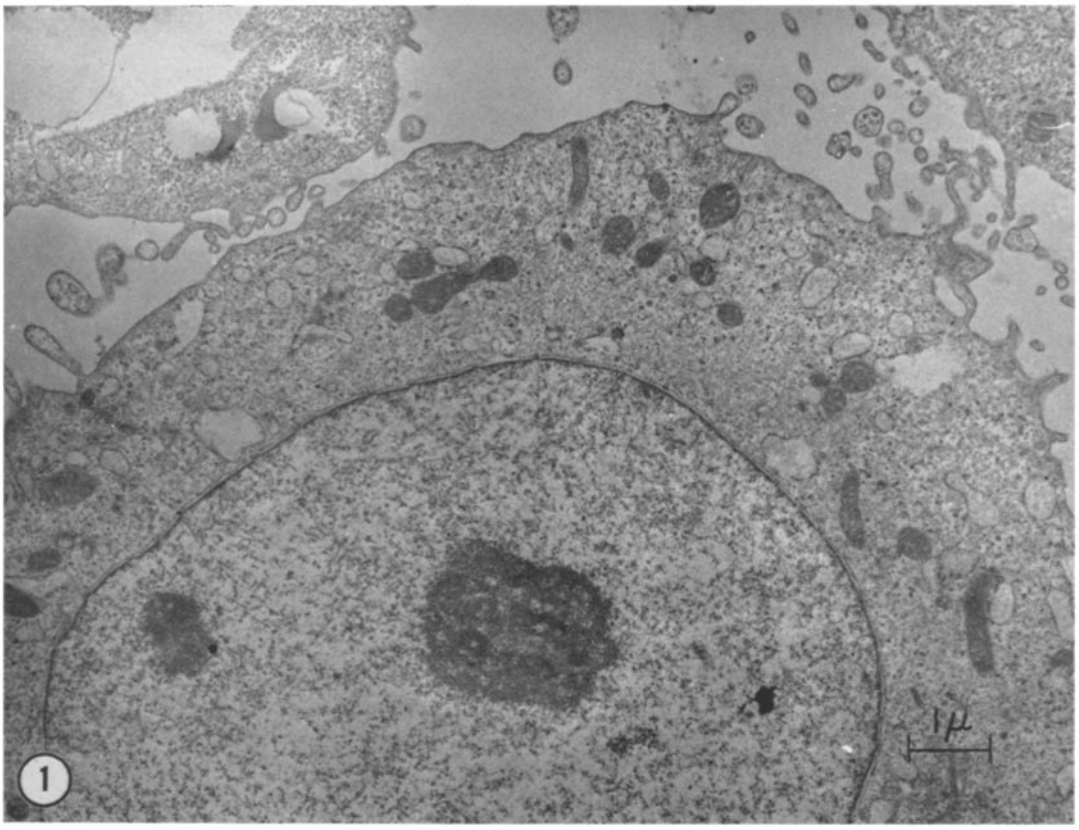

FIGURE 1 Portion of HeLa Cell after triple wash in TM saline. Note characteristic microvilli, many of which contain ribosomes. × 10,000.

Labeling of Plasma Membranes with Radioantibody

Normal and immune horse globulin were prepared by ammonium sulfate precipitation, and labeled with ^{131}I and ^{125}I, respectively, by the method of Tanigaki et al. (17). A cell pellet of 6×10^8 HeLa cells was washed three times by suspension in 100 ml of TM-saline, pH 7.4, and centrifugation at 1,000 rpm for 5 min, 20°C. 5 ml of normal horse serum which had been complement-inactivated by heating at 56°C for 45 min were added to the cell pellet, suspended in 10 ml of TM-saline, pH 7.4. A solution containing 10 mg of ^{125}I-labeled immune globulin, 10 mg of ^{131}I-labeled normal globulin, and 10% v/v horse serum was similarly inactivated and added to the cells. The mixture was incubated for 15 min at room temperature with occasional agitation, then washed four times by suspension in 100 ml of TM-saline and centrifugation at 1,000 rpm for 5 min, 20°C. Cell viability after this procedure was over 99%.

The specific fixation of immune globulin to the plasma membranes, corrected for nonspecific adsorption, was then determined for the whole cells and for various fractions during the isolation procedure by double channel counting in a gamma spectrometer.

Electron Microscopic Procedures

Cells or ghosts were fixed with 3% glutaraldehyde–1% osmium tetroxide and embedded in Epon.

Pellets of material from sucrose density gradients were fixed and alcohol-dehydrated in the cellulose nitrate tubes in which they were pelleted. The pellets were then removed, cut into strips, treated with three changes of propylene oxide, and flat-embedded in Epon. Since the different structures in the pellets tended to stratify during centrifugation, depending on their diameter and density, the pellet strips were oriented in the Epon block to give sections parallel to the radius of sedimentation. Sections were stained with lead citrate and uranyl acetate and observed in the Siemens Elmiskop 1A.

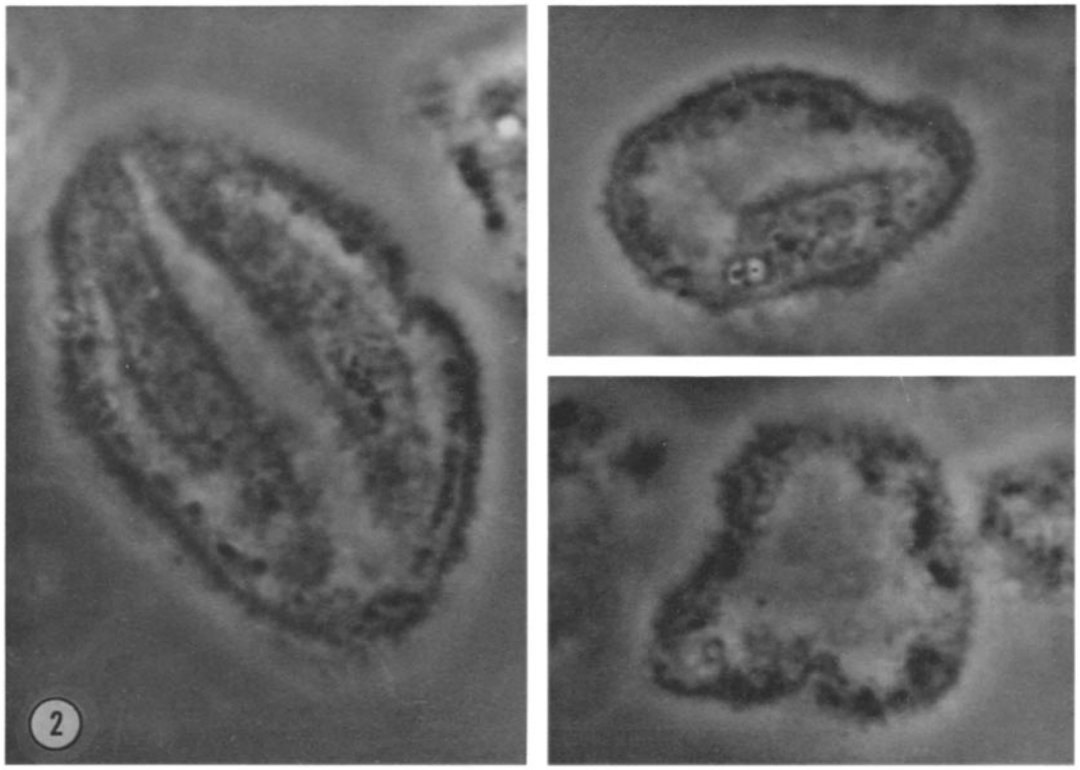

FIGURE 2 Ghosts produced by treatment of hypotonically swollen cells with the Dounce apparatus. Rents and holes in the plasma membranes are shown, through which the nuclei and most of the cytoplasm have been extruded. × 2,500.

RESULTS

Hypotonic Treatment and Cell Rupture (Step 1)

Fig. 1 is an electron micrograph of a portion of a HeLa cell from viable-frozen stock which was thawed and washed three times with TM-saline pH 7.4. The plasma membrane is characteristically formed into numerous microvilli. In the Dounce apparatus, liquid shear causes the plasma membranes of the hypotonically swollen HeLa cells to burst, leaving rounded holes and occasionally longitudinal rents at the site of rupture as shown in Fig. 2. The intact nucleus and cytoplasmic structures are extruded through these holes and cell membrane ghosts are left. Repeated washing of the ghosts by suspension and centrifugation removes most of the intracellular material.

The three cell structures left in the washed pellet are nuclei, incompletely ruptured whole cells, and ghosts. There was no evidence of nuclear disruption in any part of the isolation procedure; thus, the problem of trapping and aggregation of plasma membrane vesicles by nucleoprotein after sonication never arose.

Ghosts (Step 2)

As seen by electron microscopy (Fig. 3), the plasma membranes of the ghosts from the 35%/40% interface of the discontinuous TM-sucrose, pH 8.6, density gradient now exhibit a very large number of microvillus-like projections. Note that some of the projections contain ribosomes in their interior. This feature serves to identify some of the vesicles derived from these projections by the sonication step which comes next.

A small number of structures, particularly mitochondria and dilated vesicles of rough endoplasmic

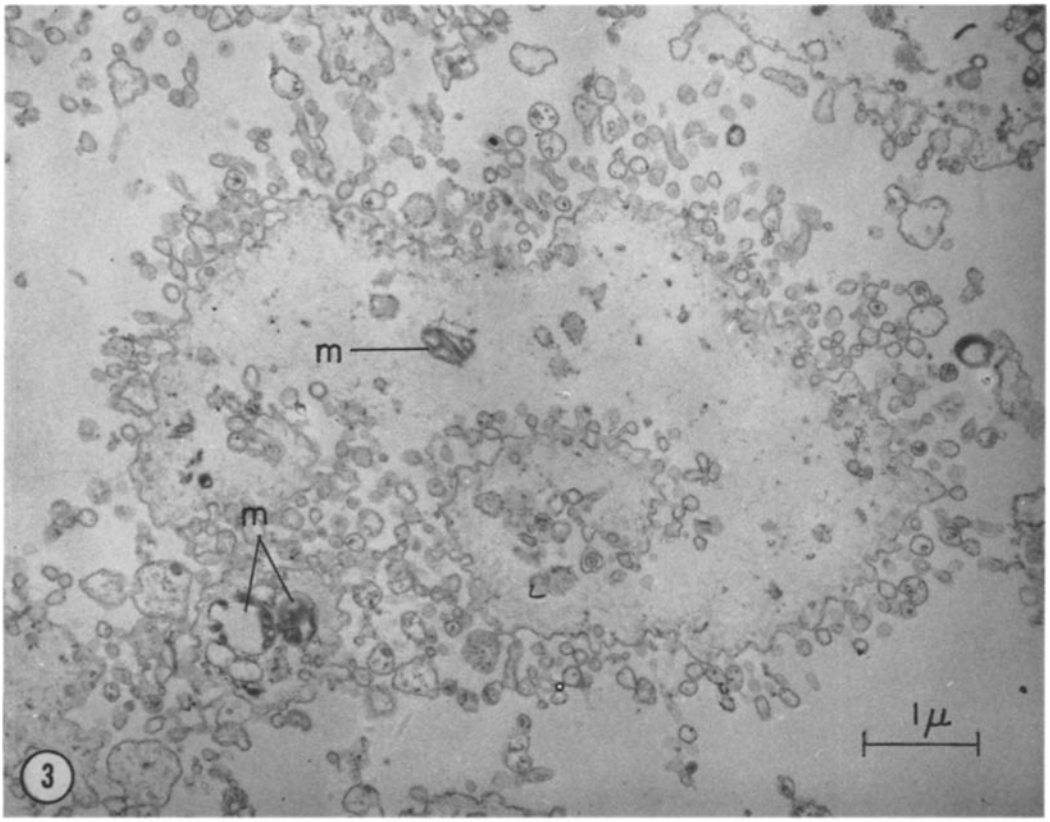

FIGURE 3 Electron micrograph of ghosts. The plasma membranes are formed into a large number of microvillus-like projections, many of which contain ribosomes. A few trapped mitochondria (*m*) are shown. Note the characteristic rolled margin of the hole in the plasma membrane. × 14,400.

reticulum, remain trapped within the ghosts and cannot be removed either by further washing (i.e. repeated centrifugation and resuspension) or by treatment with EDTA, pH 7.4, in 0.25 M sucrose. The subsequent steps of the isolation procedure are aimed at eliminating these trapped structures.

Sonicated Ghost Supernatant (Step 3)

As observed by phase-contrast microscopy, sonication of the concentrated suspension of ghosts for 5 sec causes them to disappear leaving an apparently empty field. As observed by electron microscopy, this period of sonication disrupts the plasma membranes of the ghosts into spherical vesicles 100–500 mμ in diameter, but produces no detectable effect on the remaining structures trapped inside the ghosts. In particular, the mitochondrial outer membranes remain intact. Practically all of the structures trapped in the ghosts are either much larger or more dense than the plasma membrane vesicles; they can therefore be separated from plasma membrane vesicles by the sequence of differential centrifugation followed by isopycnic centrifugation described. The differential centrifugation step removes mitochondria and other structures of similar size or larger, and the isopycnic centrifugation step separates vesicles of rough endoplasmic reticulum, as well as any remaining mitochondria, on the basis of differences in density.

It was estimated that centrifugation of the 6 ml of sonicated ghost preparation at 3,000 rpm for 15 min in the PR-2 centrifuge rotor No. 269 would pellet most structures larger than 500 mμ in diam-

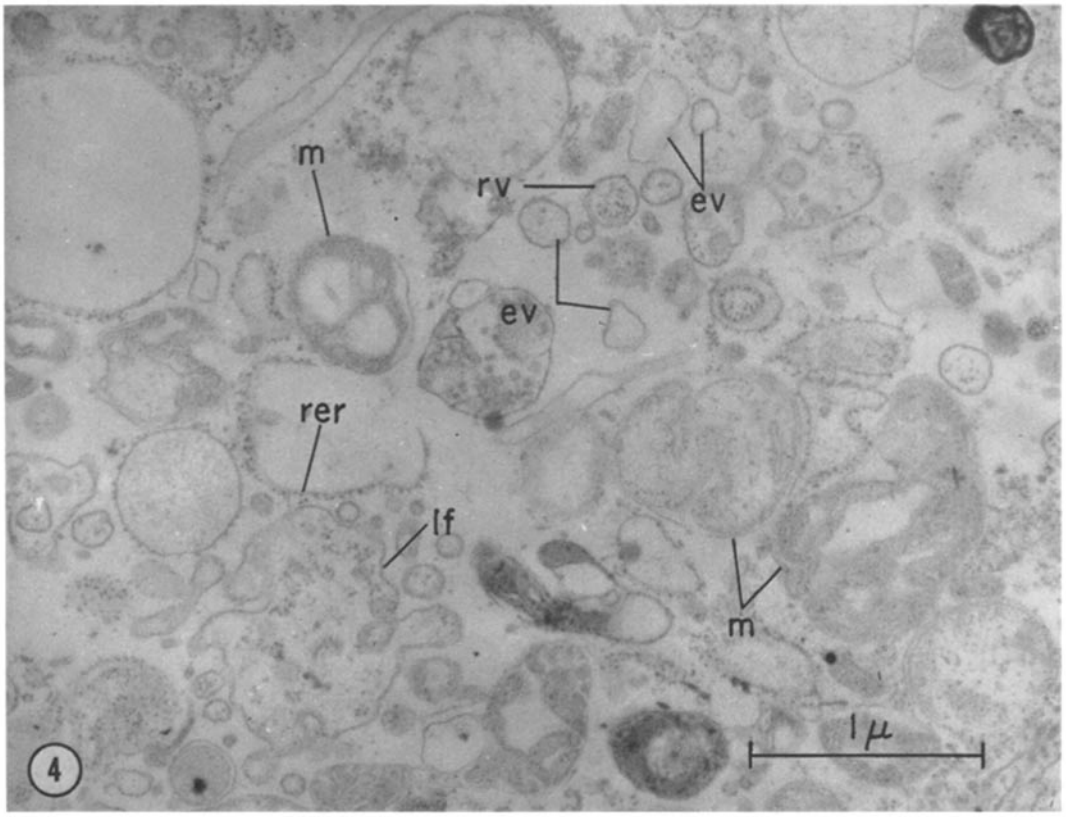

FIGURE 4 Electron micrograph of the sonicated ghost supernatant, i.e. the supernatant after differential centrifugation of a suspension of ghosts which had been sonicated for 5 sec. Vesicles derived from plasma membrane microvillus-like projections are shown; some are empty (*ev*) and some contain ribosomes (*rv*). Larger fragments of plasma membrane (*lf*), intact mitochondria (*m*), and vesicles of rough endoplasmic reticulum (*rer*) are also present. × 30,000.

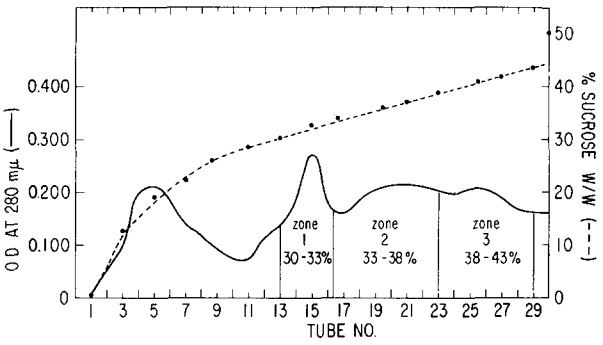

FIGURE 5 Optical density (*OD*) recording of effluent from 20–50% sucrose density gradient containing isopycnically banded material from sonicated ghost supernatant.

eter and heavier than the plasma membranes.[2] Fig. 4 shows the structures left in the supernatant (sonicated ghost supernatant) after this procedure: small smooth vesicles, many of which can be definitely identified as being derived from the microvillus-like projections of the plasma membranes because they still contain ribosomes; larger rough vesicles derived from rough endoplasmic reticulum; and mitochondria.

Isopycnic Centrifugation of Sonicated Ghost Supernatant (Step 4)

Fig. 5 is a recording of the optical density at 280 mμ versus fraction number from a 20–50% w/w sucrose gradient containing TM, pH 8.6, which was top-loaded with the sonicated ghost supernatant and centrifuged at 20,000 rpm for 16 hr, 4°C. A similar pattern occurred when the time of centrifugation was prolonged to 24 hr.

Pooled material from each of the three density zones shown in Fig. 5 was examined by electron microscopy. Fig. 6 shows that Zone 3 (38–43%) contains many mitochondria and occasional dilated vesicles of rough endoplasmic reticulum. Zone 2 (33–38%) (Fig. 7) consists of three types of structures: smooth vesicles, many of which contain trapped ribosomes; occasional rough vesicles; and larger smooth vesicles. Zone 1 (30–33%) (Figs. 8 and 9), which constitutes the symmetrical peak in the optical density record, consists of empty smooth vesicles of similar size. Vesicles containing ribosomes are occasionally found. Considering that practically the only smooth membrane structures besides plasma membranes that could be seen within the ghosts were mitochondria and, occasionally, what appeared to be lysosomes, and that these structures appear in Zone 3 of the gradient, we conclude that Zone 1 consists principally of plasma membrane vesicles. The modal density of this peak was 1.137 (average of 8 experiments, σ = 0.002). The location in the heavier Zones 2 and 3 of plasma membrane vesicles containing ribosomes can be attributed to the greater density contributed by the ribosomes.

The Use of Radio-Iodinated Immune Globulin as a Plasma Membrane Marker

GENERAL COMMENTS: In addition to antibodies to plasma membrane, the immune horse globulin prepared against viable HeLa cells undoubtedly contained antibodies to a number of intracellular structures. Fixation of radiolabeled antibody to the plasma membrane alone was achieved by incubating viable cells with the radiolabeled, complement-inactivated immune globulin for 15 min, followed by thorough washing by repeated suspension in TM saline, pH 7.4, and centrifugation. During incubation, any antibodies to intracellular structures, being macromolecules, could not penetrate through the plasma membrane of the viable cells. Ryser (13) has reported that cultured sarcoma S-180 cells take up ^{131}I-labeled human serum albumin at the rate of 10^5 molecules per cell per hour. Under the conditions of our ex-

[2] In estimating the speed and duration of centrifugation required to pellet a particle of given size and density, it is convenient to express the sedimentation coefficent of the particle, S', in units of centimeters per g-hour:

$$S' = \frac{a^2(Dp - Dm)}{18eta} (3600\ (980)$$

where a is the diameter of the particle in centimeters, Dp the density of the particle, Dm the density of the medium, and η the viscosity of the medium in poises. The reciprocal of S', when multiplied by the number of centimeters the particles must travel to reach the pellet, gives the total g-hours required.

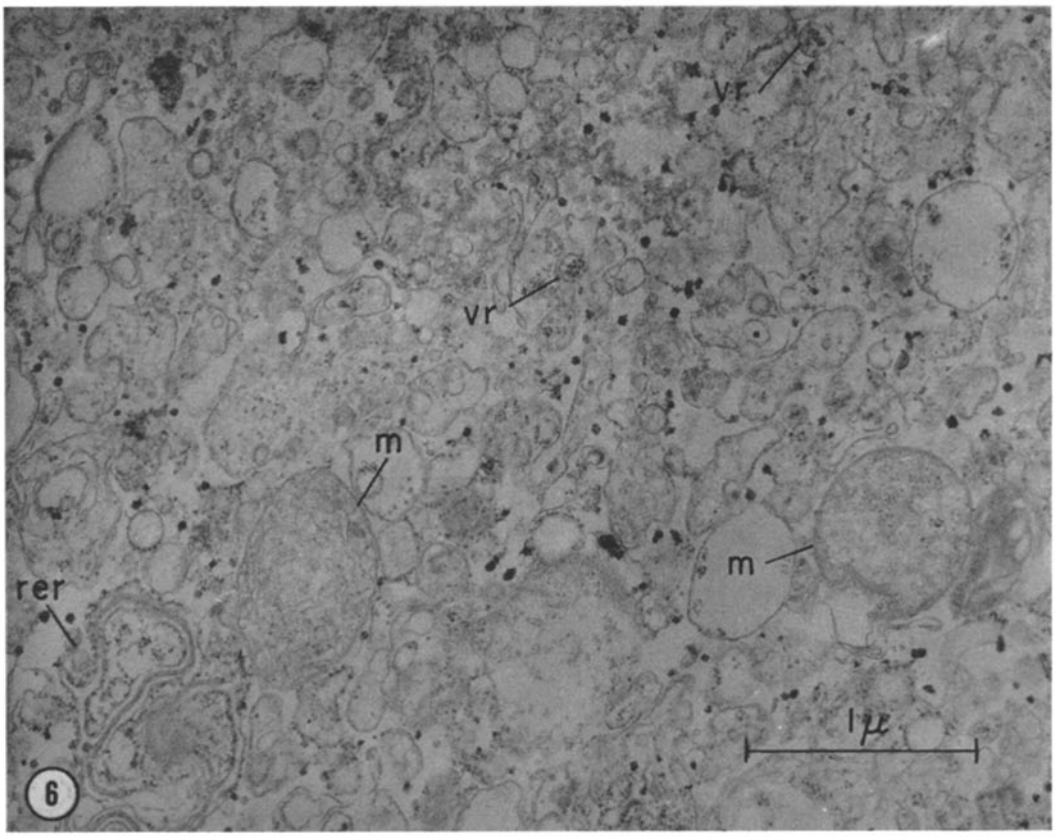

FIGURE 6 Electron micrograph of material from Zone 3 (38–43%). Shown are plasma membrane vesicles derived from microvillus-like projections which contain many ribosomes (*vr*), intact mitochondria (*m*), and vesicles derived from rough endoplasmic reticulum (*rer*). × 30,000.

periment, which included dilution of the labeled globulin with 30% horse serum, this rate of uptake could have accounted for no more than 0.01% of the labeled globulin fixed by the cells. All sera used in the experiment were complement-inactivated at 56°C for 45 min to prevent complement-dependent cytotoxic action from damaging the plasma membranes. The absence of damage was shown by the fact that more than 99% of the cells excluded Trypan blue dye after incubation with the immune globulin and washing. Using time-lapse photography, Carey and Pettengill (2) found that when HeLa cells were incubated with complement-inactivated anti-HeLa cell rabbit serum, the only visible effect was a decrease in the undulations of the plasma membrane, which began after 10 min. This effect was dependent on the concentration of antiserum and was reversible.

LACK OF A DIFFERENCE IN NONSPECIFIC ADSORPTION OF NORMAL AND ANTI-HeLa CELL IMMUNE GLOBULIN PREPARATIONS: Since the correction for nonspecific adsorption of antibody globulin in the paired-label technique depends on the absence of a difference in nonspecific adsorption between normal globulin and anti-HeLa immune globulin, a test for such a difference was made by incubating the paired-label mixture with mouse L cells which had been suspension-cultured in the same type of medium that had been used to culture the HeLa cells. The results are shown in Table I. There was no difference in the amount of nonspecific adsorption of the normal and immune globulin to the L cells. The results also show that in the immune globulin there were no detectable antibodies to fetal calf serum from the HeLa culture medium. A small amount of fetal calf serum could have remained adsorbed to the washed cells that were used for immunization.

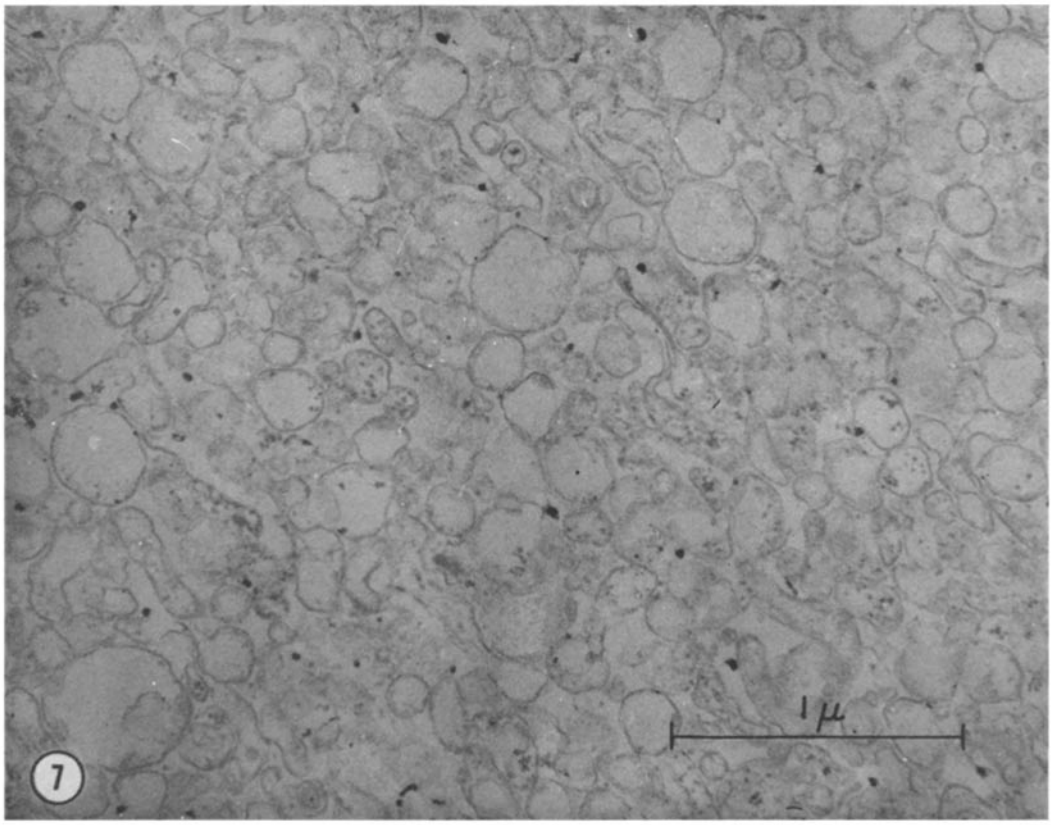

FIGURE 7 Electron micrograph of material from Zone 2 (33–38%). Smooth vesicles of various sizes, some containing ribosomes, are seen. × 38,000.

ISOLATION OF RADIOANTIBODY-LABELED PLASMA MEMBRANES. Table II shows the specific fixation of antibody, in milligrams of antibody fixed per milligram of sample protein, at each step of the plasma membrane isolation procedure. The specific fixation of the final plasma membrane fraction is increased 49-fold over that of the original whole cells. Table II also shows the yield of total sample protein in each isolation step. The yield of labeled plasma membrane protein was 0.13% of the starting whole cell protein. Because of the protein contributed by the fixed antibody, this value is higher than the more usual yield of unlabeled plasma membrane protein connected with the experiment illustrated in Fig. 10: 0.037% of starting whole cell protein.

Fig. 11 shows the specific fixation of ^{125}I-labeled antibody in each fraction of the sucrose gradient after isopycnic centrifugation (Step 4). The peak of specific activity appears at 35.1% (density = 1.154), which represents a shift from the peak value of 31.68 (density = 1.137) when radiolabeled antibody was not used. The extra weight contributed by the antibody fixed to the plasma membrane vesicles, which had to be considerable to give a specific fixation of 0.5, is a likely explanation for the shift.

ENZYME AND RNA ANALYSES OF THE PLASMA MEMBRANE FRACTIONS

ALKALI-ACTIVATED OUABAIN-SENSITIVE ATPase: Wallach and Ullrey (19) have reported on the use of a magnesium dependent, sodium- and potassium-stimulated, ouabain-sensitive ATPase as a plasma membrane marker. The enzyme is thought to be associated with the activated transport of sodium and potassium ions across the plasma membrane (8). The specific activity of this enzyme in different regions of the continuous sucrose density gradient containing isopycnically

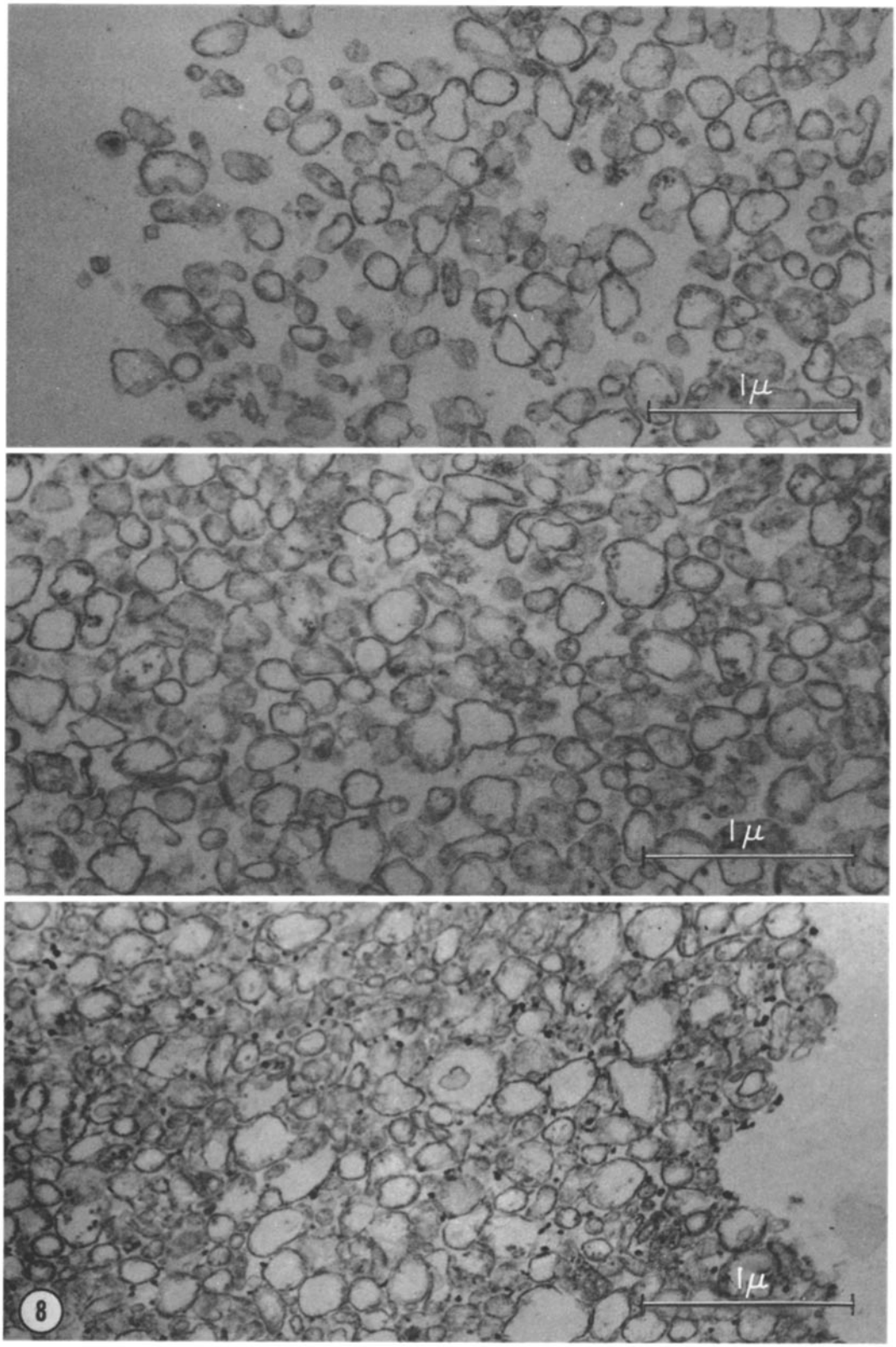

FIGURE 8 Electron micrographs of material from the top, middle, and bottom of a pellet from Zone 1 (30–33%). The top border of the pellet is at the upper left; the bottom border is at the lower right. × 33,000.

banded sonicated ghost supernatant (Step 4, Materials and Methods) is shown in Table III. The highest specific activity of Na-K ATPase is found in the region closely corresponding to Zone 1 (30–33%); this finding supports the conclusion already reached on morphological grounds that this fraction consists principally of plasma membrane vesicles. The relatively high specific activity

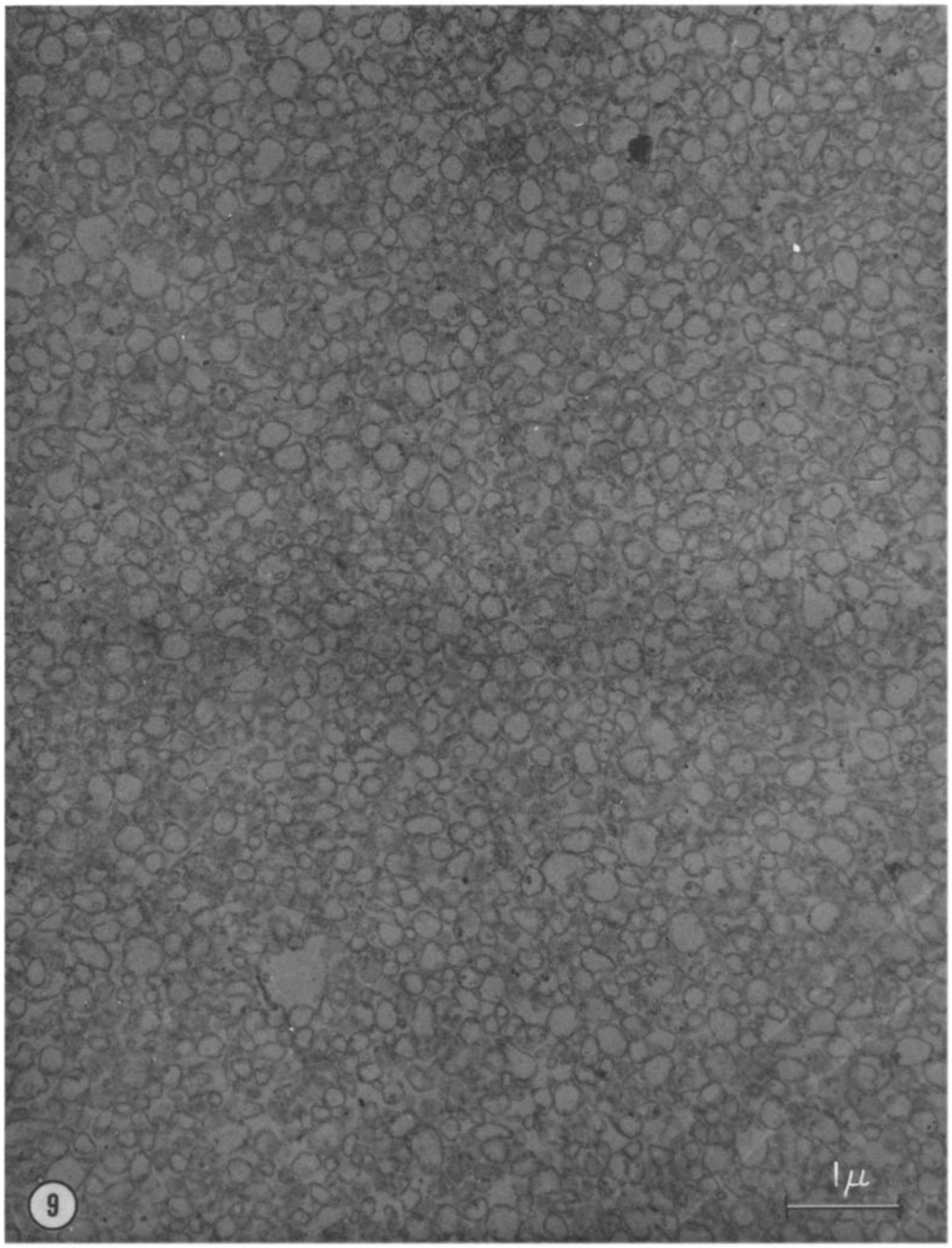

FIGURE 9 Electron micrograph of material from middle of a pellet from Zone 1 (30–33%). × 16,200.

in heavier portions of the gradient reflects the electron microscopic finding that many plasma membrane vesicles containing ribosomes were found in these heavier portions. The lack of complete inhibition of plasma membrane Na-K ATPase by ouabain was also found by Wallach and Ullrey for Ehrlich ascites tumor cells (19).

NADH-CYTOCHROME *c* REDUCTASE: The three major possible sources of contamination of the plasma membrane fraction, deriving from the relatively small amount of residual cytoplasmic material trapped inside of the ghosts, were (*a*) mitochondrial outer membranes, (*b*) smooth endoplasmic reticulum, and (*c*) rough endoplasmic reticulum from which the ribosomes might have been detached by the short period of sonication. All three of these structures are known to be associated with NADH-cytochrome *c* reductase (12, 14). As shown in Table IV, the plasma membrane fraction (Zone 1) exhibited an NADH-cytochrome *c* reductase specific activity which was 13.8% that of Zone 3, in which mitochondria and rough endoplasmic reticulum were found.

As an estimate of the per cent by weight of contamination of Zone 1 with mitochondrial outer membranes and endoplasmic membranes, 13.8% represents a maximum, since Zone 3 also contained ribosomes and mitochondrial inner membranes which do not have NADH-cytochrome *c* reductase activity. In addition, the possibility that the plasma membranes themselves possess a small degree of NADH-cytochrome *c* reductase activity cannot be ruled out (4). Smooth endoplasmic reticulum was uncommonly observed in the electron micrographs of the ghosts.

TABLE I

Data Showing Lack of a Difference in Nonspecific Fixation to 5 × 10⁶ Mouse L Cells of Anti-HeLa Cell Immune Horse Globulin and Normal Horse Globulin

	^{125}I-labeled immune globulin	^{131}I-labeled normal globulin
μg labeled protein in 50:50 mixture	190	190
μg labeled protein fixed to cells	0.334	0.320

TABLE II

Isolation of HeLa Plasma Membranes Monitored with Radiolabeled Antibody
Paired Label Mixture: 10 mg immune globulin labeled with ^{125}I; 10 mg normal globulin labeled with ^{131}I. Sample: 5 × 10^8 cells

	Total sample protein	Immune globulin fixed	Normal globulin fixed	Specific fixation mg antibody fixed/mg sample protein
	mg	*μg*	*μg*	
Whole cells after antibody fixation:	196	2.80	0.596	0.0112
Dounce homogenization:				
Low speed supernatant	93.2	0.820	0.144	0.0073
Low speed pellet	97.1	2.16	0.345	0.019
Discontinuous sucrose gradient:				
35%/40% interface (ghosts)	5.05	0.632	0.045	0.116
40%/45% interface	7.48	0.270	0.064	0.028
45% (pellet)	12.6	0.245	0.086	0.013
Continuous sucrose gradient:				
33–36%*	0.241	0.132	0.003	0.549
36–41%	0.198	0.070	0.002	0.354
41–46%	0.164	0.027	0.002	0.168

* This fraction contained the plasma membrane peak found between 30-33% in other experiments. The shift of the peak to higher density values is attributable to the presence of bound antibody.

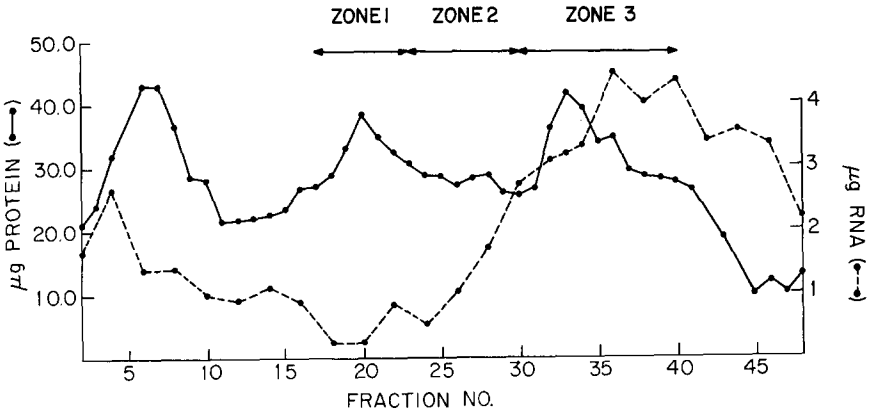

FIGURE 10 Protein and RNA contents of fractions from 20–50% sucrose density gradient containing isopycnically banded material from sonicated ghost supernatant. Starting whole cell protein: 0.63 g (1.5×10^9 cells). Sample protein placed on gradient: 2.88 mg. Recovery, Zone 1: 0.224 mg, Zone 2: 0.193 mg, Zone 3: 0.326 mg.

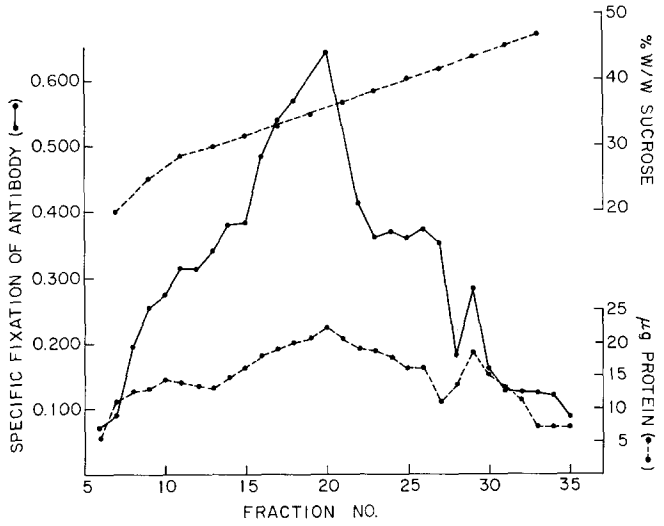

FIGURE 11 Specific fixation of anti-plasma membrane antibody and protein content of fractions from 20–50% sucrose density gradient containing isopycnically banded material from sonicated ghost supernatant.

RIBONUCLEIC ACID: Fig. 10 shows that the RNA content of the plasma membrane fraction was less than 3 mg per 100 mg of protein. The amount of RNA is increased in heavier parts of the gradient because of the presence of ribosomes either attached to rough vesicles or trapped within plasma membrane vesicles.

DISCUSSION

The method of ghost isolation which constitutes the first two steps of the plasma membrane isolation procedure described is a modification of one described by Warren et al. (20). The principal changes were: repeated washing of the low-speed pellet from the Dounce homogenate to remove as much material trapped in the ghosts as possible; bottom loading, rather than top loading, of the pellet material containing ghosts on a single discontinuous sucrose density gradient; and the routine use of washing and gradient solutions at pH 8.6, rather than at pH 7.4. The density gradient and ATPase marker techniques used in establishing some of the subsequent steps of the method are derived from the work of Wallach and coworkers (7, 18, 19).

Starting the isolation procedure by homogenizing whole HeLa cells, without first processing them into ghosts, would have posed the problem of

TABLE III

Sodium-Potassium-Stimulated ATPase in Material from Sucrose Density Gradient Fractions after Isopycnic Banding of Sonicated Ghost Membranes

1 ml mixtures contained 70–100 μg of sample protein, 20 mM Tris, pH 8.4, 0.5 mM ATP, 0.5 mM Mg^{++}, 0.1 mM EDTA, 100 mM K^+, 5 mM K^+, 0.5% ouabain. Incubated 30 min at 37°C. Activity given in micromoles Pi liberated per hour per milligram of sample protein.

Gradient fraction	A Mg^{++}	B Mg^{++}-Na^+ -K^+	C Mg^{++}-Na^+ - K^+ ouabain	B—A Na^+ - K^+ ATPase	Inhibition by ouabain $\frac{B-C}{B-A} \times 100$
% sucrose					%
29–32	1.53	3.12	2.40	1.59	45.3
32–34	1.76	3.19	2.42	1.43	69.2
34–38	1.96	2.96	2.46	1.00	50.0
38–40	1.95	2.85	2.50	0.90	38.9
40–45	1.83	2.52	2.44	0.69	8.6

TABLE IV

NADH-cytochrome c Reductase in Material from Sucrose Density Gradient Fractions after Isopycnic Banding of Sonicated Ghost Membranes

Reductase: 1 ml reaction volume contained 40–60 μg of sample protein, 0.05 M phosphate buffer, pH 7.4, 0.033% bovine cytochrome *c*, 2×10^{-4} M NADH, 0.001 M KCN. Activity is expressed as the change in optical density per minute at 550 mμ (ΔE_{550}), between 15 and 135 sec after addition of the sample, per milligram of sample protein.

Gradient fraction	Reductase ΔE_{550}	% of Zone 3
% sucrose		
Zone 1 (30–33%)	0.264	13.8
Zone 2 (33–38%)	0.934	49.0
Zone 3 (38–45%)	1.910	100

separating the plasma membrane microvesicles from other morphologically indistinguishable smooth vesicles derived from a number of organelles within the cell: mitochondrial outer membranes, smooth endoplasmic reticulum, lysosomal membranes, Golgi membranes, endocytotic vacuolar membranes, and nuclear inner membranes. In working with Ehrlich ascites carcinoma cells ruptured by nitrogen cavitation, Kamat and Wallach (7) dealt with this problem by demonstrating, through the use of enzyme and antigen markers, that in gradients of polysucrose (Ficoll) the bulk of the microsomal membranes undergo a greater change in density in the presence of divalent cations than do the plasma membrane vesicles; this permits separation of the latter by centrifugation in a discontinuous Ficoll gradient.

In the procedure described here, most of the intracellular structures containing smooth membranes are removed during the initial processing of the cells into ghosts. The few remaining larger organelles, mostly mitochondria, are separated by differential centrifugation after a minimum period of sonication (5 sec) sufficient to break up the plasma membranes into microvesicles but not to disrupt the organelles. The plasma membrane vesicles are then finally purified of remaining mitochondria and rough endoplasmic vesicles by isopycnic centrifugation on a continuous sucrose density gradient.

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