

FURTHER CHARACTERIZATION OF HeLa S₃ PLASMA MEMBRANE GHOSTS

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ABSTRACT

A plasma membrane fraction of HeLa S₃ cells, consisting of ghosts, is characterized more fully. A simple procedure is described which permits light and electron microscope study of the plasma membrane fraction through the entire depth of the final product pellet and through large areas parallel to the surface. Contamination by nuclei is 0.14%, too little for DNA detection by the diphenylamine reaction. Contamination by rough endoplasmic reticulum and ribosomes is small, a single ghost containing about 3% of the RNA in a single cell. Mitochondria were not encountered. Electron microscopy also shows (a) small vesicles associated with the outer surface of the ghosts, and (b) a filamentous web at the inner face of the ghost membrane. Sodium dodecyl sulfate(SDS)-polyacrylamide gel analysis shows that of the many Coomassie Blue-stained bands, two were prominent. One, 43,000 daltons, co-migrated with purified rabbit muscle actin and constituted about 7.5% of the plasma membrane protein. The other major band, 34,000 daltons, was concentrated in the plasma membrane fraction. Two major glycoproteins detected by autoradiography of [¹⁴C]fucose-labeled glycoproteins on the gels, had apparent molecular weights of 35,000 daltons and 32,000 daltons. These major bands did not stain with Coomassie Blue. There were many other minor glycoprotein bands in the 200,000- to 80,000-dalton range.

Ouabain-sensitive, Na⁺,K⁺-adenosine triphosphatase (ATPase) activity of the ghost fraction is purified 9.1 (± 2.2) times over the homogenate; recovery of the activity is 12.0 (± 3.8)% of the homogenate. Enrichment and recovery of fucosyl-glycoprotein parallel those for ouabain-sensitive Na⁺,K⁺-ATPase activity. Fucosyl glycoprotein is recovered more than the enzyme activity in a smooth membrane vesicle fraction probably containing the bulk of plasma membrane not recovered as ghosts.

KEY WORDS plasma membrane ·
purification · fucose glycoproteins ·
adenosine triphosphatase · electron microscopy

The first published accounts of plasma membrane fractions from HeLa cells are those of McLaren et

al. (28) and Philipson et al. (33), who used monolayer cultures. Subsequently, such fractions were obtained from spinner cultures of HeLa cells, by a variety of methods (4, 6, 7, 19, 20). Some publications have included morphologic descriptions of the ghosts in the isolated fractions (4,

6, 19, 20). The increase in ghost number in the fraction, as compared with cell number in the homogenate, is a measure of plasma membrane enrichment. Enzyme "markers" show this as well (12). One of these is ouabain-sensitive Na^+ , K^+ -adenosine triphosphatase (ATPase) activity. Johnsen et al. (20) demonstrated the enrichment of this activity in a ghost-enriched fraction of HeLa S_3 cells (made by a procedure very similar to ours). We confirm their observations, although the extent of enrichment of the activity differs from theirs. In addition, (a) we establish the copurification of fucosyl glycoprotein with the ouabain-sensitive Na^+ , K^+ -ATPase activity; (b) we present a novel method by which thorough light and electron microscope evaluation of the entire fraction can be made; and (c) we show a filamentous web below the tripartite plasma membrane of the ghosts which contain both actin (or an actinlike material) and myosin (or a myosinlike material), with the actin constituting about 13% of the total protein of the ghosts.

Characterization, as reported here, of the ghosts purified by a rapid procedure utilizing one cycle of zonal centrifugation is a continuation of earlier work on this fraction (2, 4), and is important for proper interpretation of data on the assembly of fucosyl glycoproteins and their turnover in plasma membranes prepared by this procedure (2, 3, 45). The present preparations were not made in the presence of iodoacetate or azide, as in previous publications (1, 4); thus, reliable measurements of enzyme activity is possible.

MATERIALS AND METHODS

Cells and Radioactive Labeling

Stock and experimental HeLa S_3 cells were grown at 37°C in Eagle's minimal essential medium (14) in Earle's suspension powder (Grand Island Biological Co., Grand Island, N.Y., Cat. #F-14) in the absence of antibiotics. The final glucose concentration was 2 g/liter, instead of half this amount as stated under this catalog number. The growth medium was supplemented with 3.5% calf serum, 3.5% fetal calf serum (Grand Island Biological Co.) and 1% glutamine. In these conditions, cells grew logarithmically from 10×10^4 cells/ml to 100×10^4 cells/ml, with a generation time of 23 h. Cells were tested routinely for *Mycoplasma* contamination by plate culturing (2) and by Levine's enzymatic assay (25); by both these criteria they were free of such contamination. The labeled compounds used, [1- ^{14}C]-L-fucose (48.66 mCi/mmol) and [6- ^3H]-L-fucose (13.4 Ci/mmol), were purchased from New England Nuclear, Boston, Mass.

Isolation and Purification of HeLa Cell Plasma Membrane Ghosts

Cells were grown to a density of 50×10^4 /ml. They were harvested from 200 ml of culture by low-speed centrifugation (800 g, min). They were washed once in 200 ml and twice in 40 ml of Earle's balanced salt solution at 4°C, pH 6.8 (adjusted on the day of the experiment). Plasma membranes were prepared by one cycle of zonal centrifugation according to the procedure of Atkinson and Summers (4), described in detail elsewhere (1), except for the omission of iodoacetate and azide. Generally, four discontinuous gradients were used to isolate plasma membranes from 1×10^8 cells. However, in some experiments, up to 8×10^8 cells were utilized, necessitating 32 individual gradients. In a few experiments, plasma membranes were further purified by banding them isopycally once, on a continuous sucrose gradient made from 18 ml of 20% wt/wt sucrose, 18 ml of 50% wt/wt sucrose in 10 mM Tris-HCl pH 8, centrifuging for 16 h at 25,000 rpm in the SW27 rotor. The membranes purified by one cycle of zonal centrifugation from up to 2.7×10^8 cells were loaded on one such gradient. The plasma membranes banded homogeneously at 1.16 g/ml. Radioactivity and protein determinations were performed as previously described (2).

Enzyme Determinations

Ouabain-sensitive Na^+ , K^+ -ATPase was measured by the method of Jorgensen et al. (21), as described below. Because enzyme activities were low and variable in the HeLa homogenates, sodium deoxycholate (DOC) was used to activate the enzyme. The detergent, in addition, would be expected to overcome differential enzyme inaccessibility. Cell fractions and homogenates, containing 350 μg of protein, were preincubated with 0.06% DOC (the optimal amount for this protein concentration), 2 mM EDTA, 25 mM imidazole buffer, pH 7.0, in a final volume of 1.0 ml, for 30 min at room temperature. 150–200 μl of the preincubated fractions and homogenates were then incubated in a total volume of 1.0 ml consisting of 50 mM Tris-HCl pH 7.4, 1.0 mM EDTA neutralized to pH 7.0, 3.0 mM Tris-ATP (Sigma Chemical Co., St. Louis, Mo.), 100 mM NaCl, 20 mM KCl, and 3.0 mM MgCl_2 . In the tubes used to determine the ouabain-sensitive ATPase, 1.0 mM ouabain octahydrate (Sigma Chemical Co.) was present. The mixtures were incubated for 10 min at 37°C and the reaction was terminated with 0.2 ml of 25% trichloroacetic acid (TCA). Released inorganic phosphorus was determined by the molybdate method of Martin and Doty as described by Lindberg and Ernster (26). Under these conditions, the enzyme reaction was linear for 30 min. Greater reproducibility was achieved when the samples were preincubated for 2 min at 37°C and kept in ice until the addition of ATP. Ouabain-sensitive Na^+ , K^+ -ATPase activity was taken to be the activity inhibited by 1.0

mM ouabain; as the Tables show, this activity was 20–30% of the total ATPase activity in homogenates and more than 80% in the plasma membrane.

DNA and RNA Determinations

The content of DNA and RNA was determined in cell homogenates and plasma membrane fractions by the method of Volkin and Cohn (42). DNA and RNA were precipitated with 10% TCA, and the precipitate was washed once with 10% TCA. RNA was hydrolyzed for 16 h at 37°C with 1 N KOH, and DNA in 5% perchloric acid for 20 min at 90°C. All fractions were made up to measured volumes and aliquots were removed for analysis. DNA content was measured by the diphenylamine reaction (9) using calf thymus DNA as a standard. RNA was measured by the orcinol reaction (42) using yeast RNA as a standard.

Morphological Procedures

Plasma membrane fractions derived from 1×10^8 cells were processed for examination by light microscopy and electron microscopy. The fractions were pelleted in the plastic tubes. After removal of the supernatant fluid, the pellets were fixed in cold 2.5% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.), 0.1 M cacodylate buffer, pH 7.4, surrounded by ice. The fixative and subsequent fluids were added slowly, by Pasteur pipette, to the wall of the tube so that the pellet remained undisturbed. The fixative was removed after 30 min and the pellet postfixed with cold 1% OsO_4 , 0.1 M cacodylate buffer, pH 7.4, for 1 h on ice, followed by uranyl acetate en bloc staining for 1 h at room temperature in the dark (15, 23). Then, by a series of increasing concentrations of ethanol, the pellets were dehydrated. In 100% ethanol, the portion of the plastic tube containing the pellet was cut out with scissors, while submerged. Then, under a dissecting microscope, small squares (~0.5–0.8 mm) were carefully cut with a portion of a Wilkinson blade¹ from the area of the tube, still submerged, as indicated in Fig. 1. The cut squares were carefully separated from the plastic tube.² Subsequent handling of the squares was done in small saltcellars. The squares were treated with propylene oxide, 10 min; 1:1 propylene oxide, Epon 812, 2 h; and Epon, overnight. On the following day, they were embedded in two manners, as indicated in Fig. 1. For examination at right angle to the centrifugal force (“horizontal direction”), the squares were flat-embedded in the cover of a plastic

¹ Ms. Ana Yam has devised a simple device useful for these and other dissections under a binocular microscope. A small portion of a Wilkinson blade is attached by epoxy cement to the thinner end of a wooden chopstick.

² At this stage, small fragments of the pellet can be quickly examined by light microscopy after crushing them between slide and cover slip. A drop of 1% toluidine blue can be added.

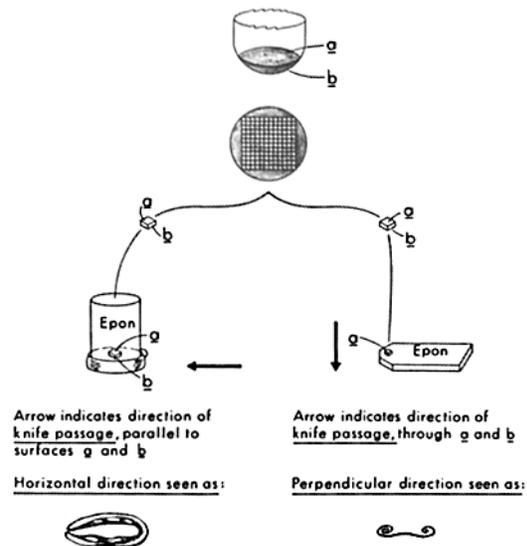


FIGURE 1 Schematic representation of embedding and sectioning of the plasma membrane pellet. Individual ghost appearances are shown diagrammatically at the bottom (cf. Figs. 2–6).

capsule (Better Equipment for Electron Microscopy, Bronx, N. Y.); the tip of the capsule had been cut off earlier to permit filling the capsule with Epon at this time. For examination in the direction of the centrifugal force (“perpendicular direction”), the squares were embedded in small rubber molds.

For light microscopy, several flat-embedded squares and several mold-embedded squares, chosen at random, were used. 1- μm and 2- μm sections were prepared, using glass knives. The sections were put on slides in drops of water and heated on a hot plate until they adhered to the slide. They were then stained with 1% aqueous toluidine blue, pH 10, for 30–60 s on a hot plate. The slides were rinsed with distilled H_2O , air-dried, dipped in xylene, and mounted with Permount (Fisher Scientific Co., Pittsburgh, Pa.). The numbers of plasma membrane ghosts and nuclei were easily enumerated in such sections (Figs. 1 and 2).

Typical areas were selected for thin sectioning of both perpendicular and horizontal orientations. Sections (pale yellow interference color) were cut with a diamond knife (DuPont Instruments, Wilmington, Del.), using the LKB Ultratome III (LKB Instruments, Inc., Rockville, Md.). These were examined in the Philips 300 microscope at 80 kV, at magnifications of $300\times$ to $21,500\times$. To photograph the entire perpendicular preparation of a fraction, sections were placed on grids (either 75-mesh or single-hole grids) coated with 1% colloidin, with or without a thin layer of carbon. The so-called scanning range of the Philips electron microscope was used. The objective aperture was replaced by the diffraction lens with a 200- μm aperture. This permits magnifications from $240\times$ to $4,500\times$.

Visualization of Actinlike and Myosinlike Materials

Antibodies against actin (a gift of Dr. Elias Lazarides, California Institute of Technology; actin isolated from chicken gizzards) were produced in rabbits and were purified by ammonium sulfate precipitation and chromatography. Actin antibody was judged to be specific for actin by immunodiffusion and complement fixation against highly purified actin (24). Purified antibody to myosin (a gift from Dr. William Gordon, Cold Spring Harbor Laboratory, New York) was monospecific as judged by immunodiffusion, immunoelectrophoresis, and crossed immunoelectrophoresis (Dr. W. Gordon, personal communication). The myosin was prepared by Dr. Gordon from chicken gizzards by affinity column chromatography using the purified antigen for coupling to Sepharose.

The cells grown on cover slips were processed by procedures based on those of Pollack and Rifkin (34) and Lazarides and Weber (24). The cover slips were placed, without rinsing, in formaldehyde (Fisher Scientific Co., reagent grade, 38%, diluted to 3.8% with phosphate-buffered saline [PBS]) at room temperature for 15 min. After a brief wash in PBS, they were treated, consecutively, for 5 min each, in water:acetone (1:1), acetone, water:acetone (1:1), and PBS. Each cover slip was covered with 10 μ l of 1:40 dilution in PBS of either rabbit antiactin or antimyosin, or of PBS alone, in a loosely covered Petri dish. The dishes were incubated in a moist incubator at 37°C for 1 h. The cover slips were then washed three times in PBS and incubated for 1 h in the same manner as previously, but covered in all cases with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Kankakee, Ill.) diluted 1:10 in PBS. The coverslips were then washed three times in PBS and once in H₂O, and mounted in Elvanol (Dupont Instruments) on glass slides. They were examined, generally the next day, with a Zeiss microscope by epi-illumination using a narrow FITC exciter filter and a 500-mm barrier-filter; 40 \times and 100 \times objectives were used. Treatment for myosin staining was the same, except that acetone treatment at -20°C was used (43). Essentially the same procedures were used with the HeLa plasma membranes, except that they were not fixed before antibody staining. Fractions prepared from 4×10^8 cells by one cycle of zonal centrifugation were split into three pellets in 15-ml Corex (Corning Glass Works, Corning, N. Y.) round-bottomed centrifuge tubes. Antibody against actin or myosin, in 100-200 μ l diluted in 1:40 in PBS, was placed on the pellets which were then gently dispersed. After a 1-h incubation in a humidified incubator, the plasma membrane fractions were washed three times in 12 ml of PBS by brief centrifugation (8,000 g, min). The pellets were then gently dispersed as before in 100-200 μ l of FITC-goat anti-rabbit IgG, diluted 1:10 in PBS, and incubated for 1 h as before. At the end of this second incubation period, the fractions were washed

three times in PBS. They were then suspended in a small quantity of Elvanol; drops of suspension were mounted between slides and cover slips.

Two negative controls and one positive control were performed in all experiments. The former consisted of (a) substituting normal rabbit gamma globulin for the specific antibody in the first incubation. (Normal rabbit serum [Arnel Products Co., Brooklyn, N. Y.] was precipitated three times with 40% saturated ammonium sulfate, and the final pellet was resuspended in the same original volume of PBS and then chromatographed on Sephadex G25; the eluate was pooled and dialyzed against PBS). It also consisted of (b) substituting PBS for antibody in the first incubation. The positive control consisted of a normal line, WI38, which shows fluorescent "cables" or "stress fibers" with either antiactin (34) or antimyosin antibodies (43, 16). Such cables were seen in all experiments (Fig. 8).

SDS-Polyacrylamide Gel Analysis

Homogenates were centrifuged at 800 g, min to remove nuclei before analysis. This step was necessary because DNA released from nuclei caused gel electrophoresis artifacts. The plasma membrane fractions analyzed were those purified by one cycle of zonal centrifugation. The marker proteins employed were actin and proteins G, L, M, and N of vesicular stomatitis virus, purified as previously described (3). The actin, freed from troponin and tropomyosin, was prepared according to Bailin and Bárány (5) as modified by Nachmias (personal communication, 1977). An acetone powder of mixed muscles of rabbit leg, from which the actin was purified, was a gift from Dr. Vivianne Nachmias, University of Pennsylvania, Pa. The materials were separated on SDS 10% polyacrylamide gels by electrophoresis in a modification of the Reid and Bielecki apparatus (37), essentially as described by Maizel (27), using the Tris-glycine discontinuous buffer system or the phosphate buffer system, where specifically stated. Identical gel analyses were made of plasma membrane fractions and homogenates labeled with [¹⁴C]fucose (0.03 μ Ci/ml) for 24 h, a labeling period known to be sufficient to equilibrate all glycoprotein-fucose with the specific radioactivity of the precursor GDP-fucose (45). Gels were dried in gel slab dryer model 224 (Bio-Rad Laboratories, Richmond, Calif.), and autoradiographed 13 days using Kodak SB-5 X-ray film. Coomassie Blue-stained gels were photographed while still in destaining solution; negatives were scanned with a Joyce-Loebl densitometer with the appropriate wedge (Joyce-Loebl, Gateshead-on-Tyne, England).

RESULTS

Morphology of Plasma Membrane Fractions

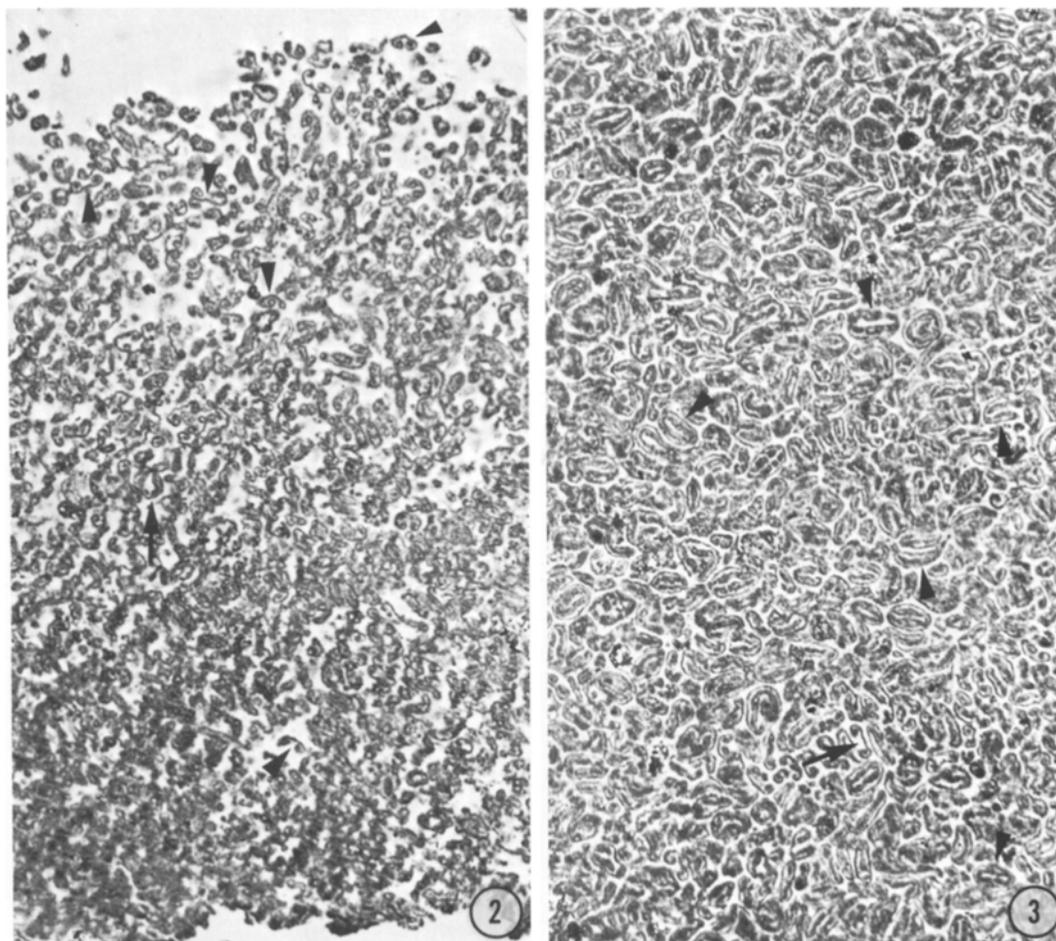
The shapes of the ghosts were different dependent upon the plane of section. In the perpendicular plane (Fig. 1), most were thin and they were

curled at each end, roughly in the shape of violin scrolls (Figs. 1, 2, 4, and 5). In the horizontal plane, boatlike shapes predominated (Figs. 1, 3, and 5). Examination of the freely floating immunofluorescent ghosts (Figs. 9-11) with oil immersion microscopy is well suited for revealing their three-dimensional aspects.

LIGHT MICROSCOPY: Light microscopy of perpendicular sections in 1- μm Epon sections (Fig. 2) permitted rapid counting of large numbers of ghosts throughout the entire depth of a given fraction, and also of contaminating whole cells or nuclei. Thus, examination of three small squares of a single fraction (Fig. 1) showed 7,350, 4,480, and 3,200 ghosts, for a total of 15,030; and 6, 0, and 15 nuclei, for a total of 21. This fraction,

then, had a nuclear contamination of 0.14%, consistent with the inability to detect DNA in the fraction by the diphenylamine procedure. No unbroken cells were seen. A very small number of unidentifiable masses were present; they might be shrunken bits of cells or small pieces of plastic from the tube (Figs. 2 and 3). Clean nuclei made by the method of Penman (32) have 3.06×10^{-5} μg of protein/nucleus (unpublished experiments of Paul H. Atkinson). From this figure and the estimation of protein per ghost, 2.71×10^{-5} μg (see below), it can be calculated that this represents a 0.16% contamination by nuclear protein, close to our morphologic enumeration.

ELECTRON MICROSCOPY: The highly purified nature of the plasma membrane fraction is



FIGURES 2 and 3 Light micrographs of Epon sections, $\sim 1 \mu\text{m}$ thick, stained with toluidine blue. $\times 370$. Fig. 2. Perpendicular direction encompassing the entire depth of the pellet. The "fiddle" shapes (arrowheads) predominate and "boat" shapes (arrow) are infrequent. Fig. 3. Horizontal direction. The "boat" shapes (arrowheads) predominate and "fiddle" shapes (arrow) are seen infrequently.

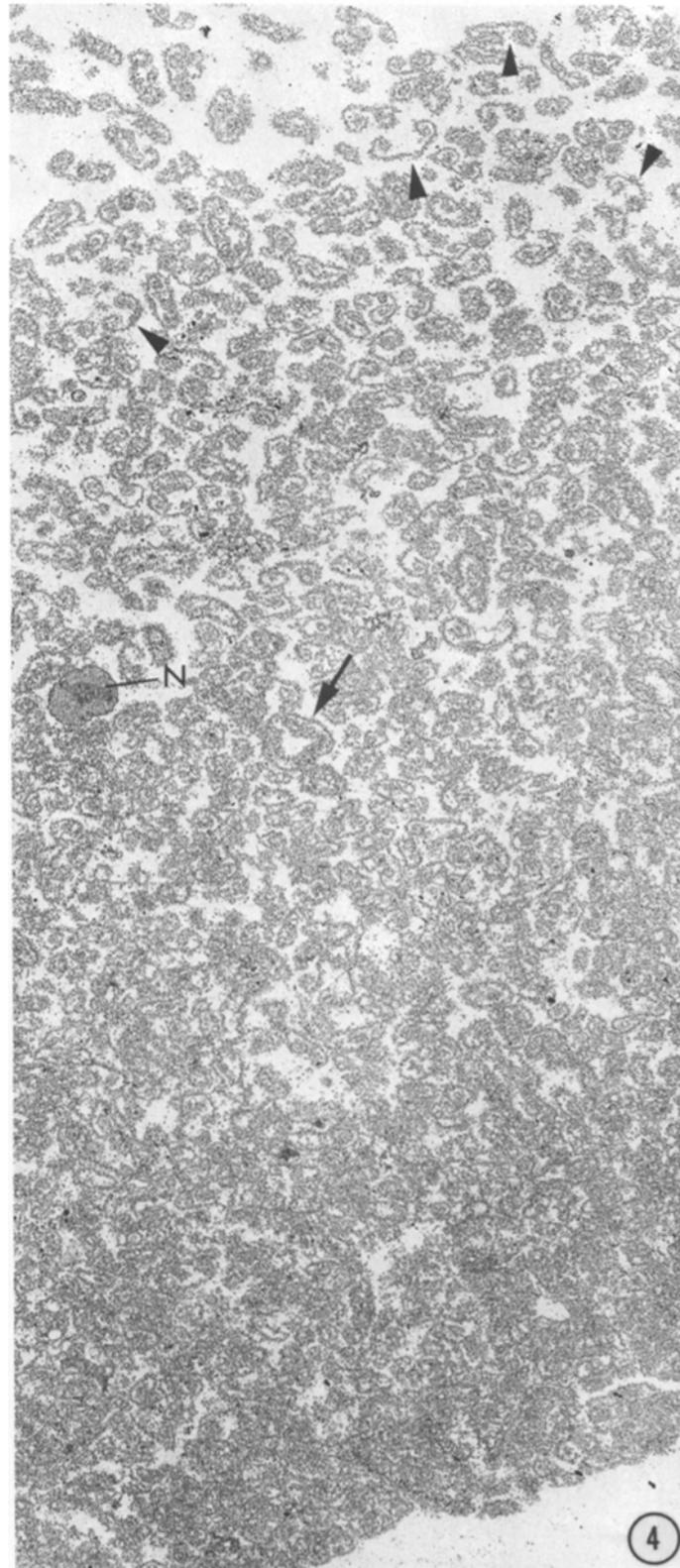


FIGURE 4 Low-magnification electron micrograph of a perpendicular section of the entire plasma membrane pellet. Arrowheads indicate the "fiddle" shapes that predominate when the pellet is sectioned in this plane. The arrow indicates a "boat" shape, seen infrequently. A rare nucleus is indicated at *N*. $\times 660$.

indicated by Fig. 4, which shows the entire depth of the pellet. Light microscope examination of the 1- μ m Epon section had revealed a single nucleus in the area, and this was chosen for thin sectioning.

With sufficiently high magnification, the tripartite plasma membrane is readily evident (not illustrated) when the area of membrane as sectioned is properly oriented with respect to the electron beam. Always associated with the inner surface of the tripartite plasma membrane is a web of filaments. The depth of the filamentous web appears variable dependent upon its relation to the plane of section (Fig. 7). In vertical sections through the plasma membrane, the web measures \sim 200 nm. From thin sections it is difficult to say much about the natures of the individual filaments. Many filaments are \sim 4 nm thick. It is not possible to discern how many of these are sections of microfilaments (5-7 nm in diameter), thicker myosin filaments, or thinner "microtrabecular" filaments (44). Results to be described below show the presence of both actinlike and myosinlike materials in the ghosts.

The ghosts show a variable number of projections appearing both as irregular pseudopods ("microvilli") and as circular or ellipsoid vesicles of variable size. Most projections show filamentous web material in their interiors, and sometimes a few ribosomes (Fig. 7) are present, as in the filamentous web of the main bodies of the ghosts.

In the many electron micrographs taken of plasma membrane fractions including perpendicular sections, from top to bottom, of the pellets and horizontal sections, not a single mitochondrion was observed. Vesicles of ribosome-studded endoplasmic reticulum (ER) are found, but these are relatively rare, as are unidentifiable smooth membrane vesicles (Fig. 7). Ribosomes are more frequent but their number is still very small. They are near the inner surface of the filamentous web (Fig. 7), but some may be closer to the tripartite membrane. However, ribosomes resting upon the inner surface of the tripartite membrane were never observed. Occasionally, ribosomes are arranged linearly as if they were portions of polyosomes.

It is possible to find vesicles that appear free, i.e., unattached to the ghosts (Fig. 7). However, it is safe to conclude that the vesicles were attached to the ghosts and that the attachments were not seen because the section is parallel to

(above or below) the ghost surfaces in these areas. At the very low centrifugal forces (8,000 *g*, min) used to sediment the ghosts, free vesicles of this size would not be expected.

Protein DNA and RNA Content of Ghosts

Protein content was $26.4 \pm 1.7 \times 10^{-5}$ μ g/cell. The total protein in one ghost calculates as $2.71 (\pm 0.58)^3 \times 10^{-5}$ μ g, as determined by ghost counting and protein determination in the fraction. The DNA of the homogenate measures 97.96 μ g/mg protein. It was not detectable in the isolated fraction, and must thus be less than 2.5 μ g/mg protein. The RNA of the homogenate measured 167 μ g/mg protein. The RNA in the isolated fraction measured 49.2 (± 5.3) μ g/mg protein. Thus, the RNA in one ghost calculates as 3.0% of cell RNA. On the basis of recovery of ouabain-sensitive Na⁺-K⁺ ATPase, the protein content per ghost was 10.2% of the whole cell.

Actin and Myosin

Plasma membrane ghosts stained specifically for antibody directed against chicken gizzard actin or myosin. The typical "boat" appearances (Figs. 9 and 11) and "fiddle" appearances (Fig. 10) are evident, dependent upon the viewing angle (see above). Ghosts which had been treated with normal rabbit gamma globulin instead of antiactin gamma globulin did not stain with FITC-goat anti-rabbit gamma globulin, nor did ghosts treated with PBS instead of antiactin before the FITC. This indicates the specificity of FITC-goat anti-rabbit gamma globulin fluorescence at the dilution used. As a positive control, stress fibers or cables, similar to those in published photographs (24), were observed in WI38 cells grown on cover slips (Fig. 8). Similarly, plasma membrane ghosts showed specific staining for myosin or myosinlike protein (Fig. 10). Stress fibers were also observed in WI38 cells similar to those shown in other cell types (43).

Plasma membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis. There were obviously many bands, and careful inspection shows that there are many bands prominent in the membranes and not in the cells, and vice versa. However, two major bands stained with Coomassie Blue were observed with apparent molecular weights of 43,000 and 34,000 (Fig.

³ This and percent variations indicated elsewhere are standard deviations of the mean.

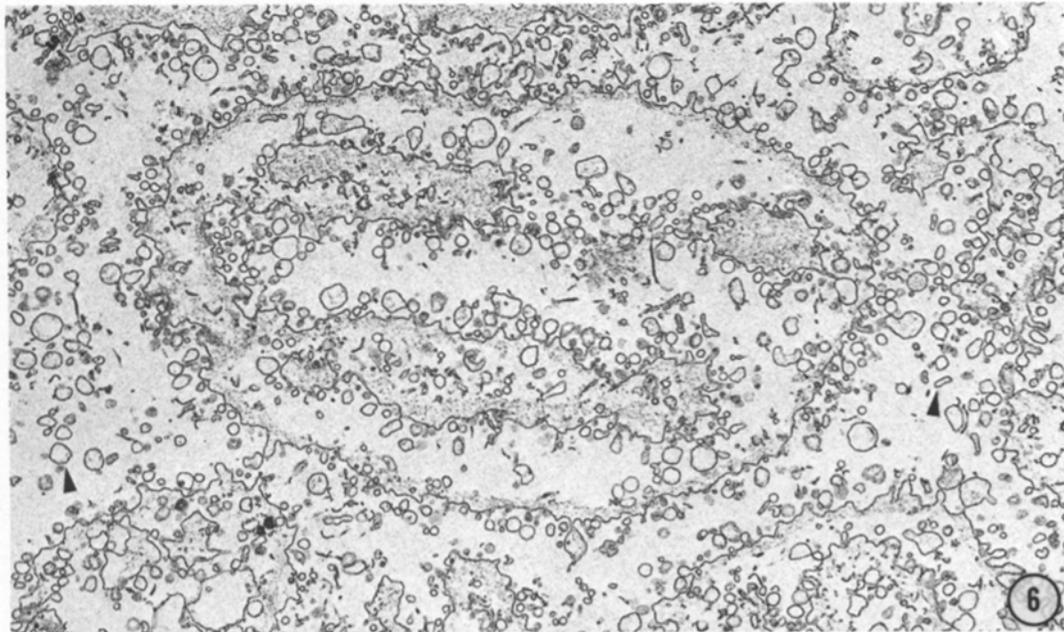
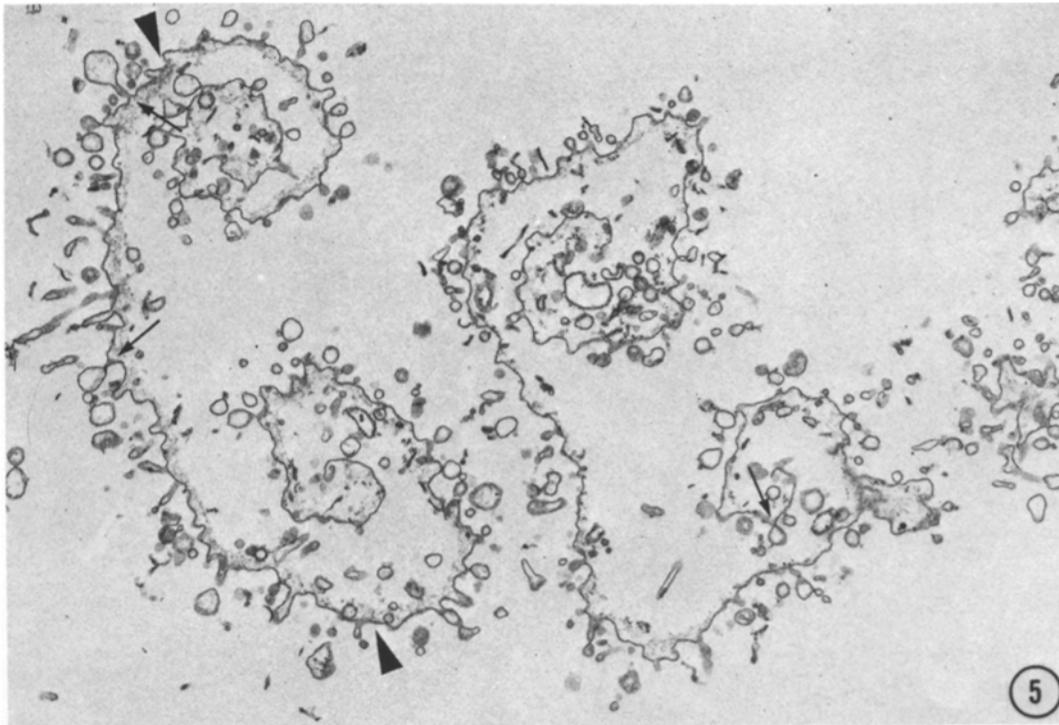


FIGURE 5 Ghosts from a perpendicular section of the plasma membrane pellet. In the two "fiddles" that fill most of the field, the plasma membrane is seen to be curled under into a scroll at each end. The network of filaments on the inner aspects of the ghosts is barely evident at this low magnification but shows clearly in enlargements (Fig. 7). There are numerous outward projections of the plasma membrane surfaces, in the form of vesicles and pseudopods. Some of the vesicles appear to be separating from the surfaces (arrows). Others seem to be free of the surfaces; these are probably attached to the surfaces in the other planes of section (see text). The two arrowheads indicate the probable plane of section of Fig. 6. $\times 10,000$.

FIGURE 6 Ghosts from a horizontal section of the plasma membrane pellet. Most of the field is occupied by a ghost with a "boat" appearance. The curled-under portion of the ghost appears separated from the outer part because the plane of section was like that indicated by the arrowheads in Fig. 5; other sectioning planes would show connections between the two parts. The two arrowheads are directed at what appear to be free vesicles, but see text for discussion of their probable attachment to the ghosts in another plane of section. $\times 7,500$.

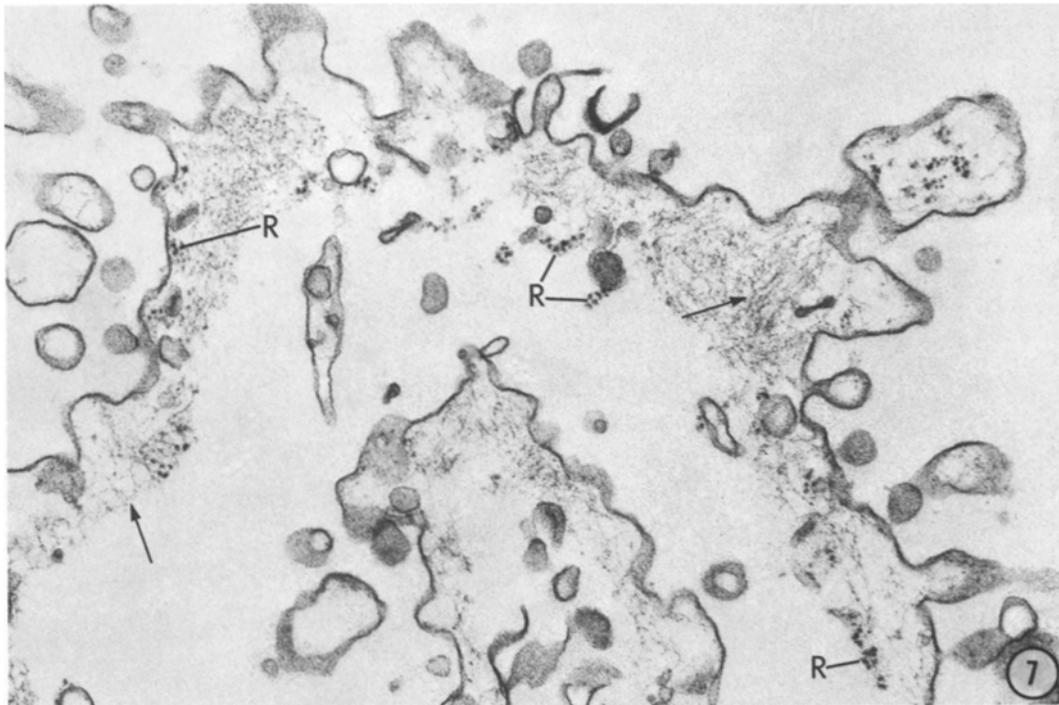


FIGURE 7 Portions of a ghost from a perpendicular section of the plasma membrane pellet. The arrows indicate the network of filaments on the inner aspect of the ghost; at higher magnification (not illustrated), the ghost shows the typical tripartite nature of plasma membranes. Some ribosomes (*R*) are seen; note that even the ribosome closest to the inner surface of the membrane (*R* at left) does not make contact with the membrane. $\times 33,500$.

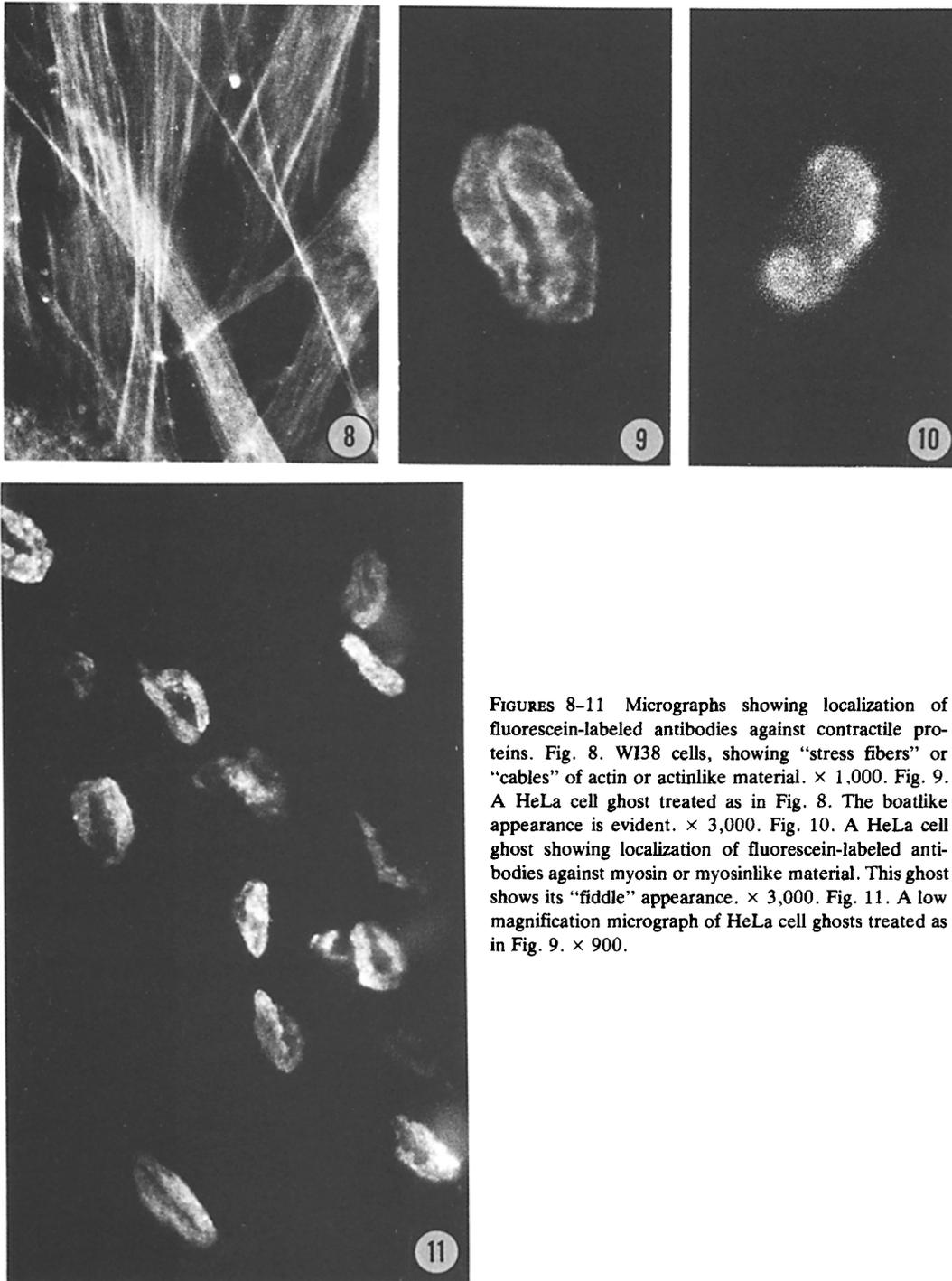
12). Densitometry indicated that the 34,000-dalton band consisted of a major species and a number of minor species. (This band migrated at $\sim 38,000$ daltons in SDS-polyacrylamide gels buffered with phosphate instead of Tris-glycine.) Autoradiography of [^{14}C]fucose-labeled glycoproteins separated by the gels showed that two bands constituted the major glycoproteins of HeLa plasma membranes (Fig. 13). The more abundant of the two was 1,000 daltons higher in apparent molecular weight than the 34,000-dalton band. The other glycoprotein was 2,000 daltons lower in apparent molecular weight. We had previously noted (4) that the major fucosyl glycoprotein band, though migrating in SDS-gel electrophoresis at a rate similar to that of one of the major protein bands, appeared to be doing so independently. Also, as expected from earlier conclusions (2), these glycoprotein bands were not associated with any prominent Coomassie Blue-stained band (Fig. 13). There were at least 20 other discernible minor glycoprotein bands ranging in apparent molecular weights from 200,000 daltons to 80,000

daltons (~ 16 bands) down to the major species of $\sim 35,000$ daltons and 32,000 daltons. The major 43,000-dalton, Coomassie Blue-stained band comigrated with actin purified from rabbit muscle. In three different plasma membrane preparations, this actinlike band constituted $7.45 \pm 0.13\%$ of the protein of the plasma membranes as determined by densitometer scanning of Coomassie Blue-stained gels from several different preparations of plasma membrane fractions.

Ouabain-Sensitive, Na^+ , K^+ -ATPase; Mg^{++} , Na^+ , K^+ -ATPase, and Fucosyl Glycoprotein

Ouabain-sensitive, Na^+ , K^+ -ATPase activity is relatively low in homogenates, leading to some variability in measurements. The use of the detergent DOC causes two- to fourfold activation of such ATPase activities in both homogenates and ghost fractions, and reduces the variability of the activities in both.

The ghost fraction purified by one cycle of rate zonal centrifugation are enriched 9.1-fold in oua-



FIGURES 8-11 Micrographs showing localization of fluorescein-labeled antibodies against contractile proteins. Fig. 8. WI38 cells, showing "stress fibers" or "cables" of actin or actinlike material. $\times 1,000$. Fig. 9. A HeLa cell ghost treated as in Fig. 8. The boatlike appearance is evident. $\times 3,000$. Fig. 10. A HeLa cell ghost showing localization of fluorescein-labeled antibodies against myosin or myosinlike material. This ghost shows its "fiddle" appearance. $\times 3,000$. Fig. 11. A low magnification micrograph of HeLa cell ghosts treated as in Fig. 9. $\times 900$.

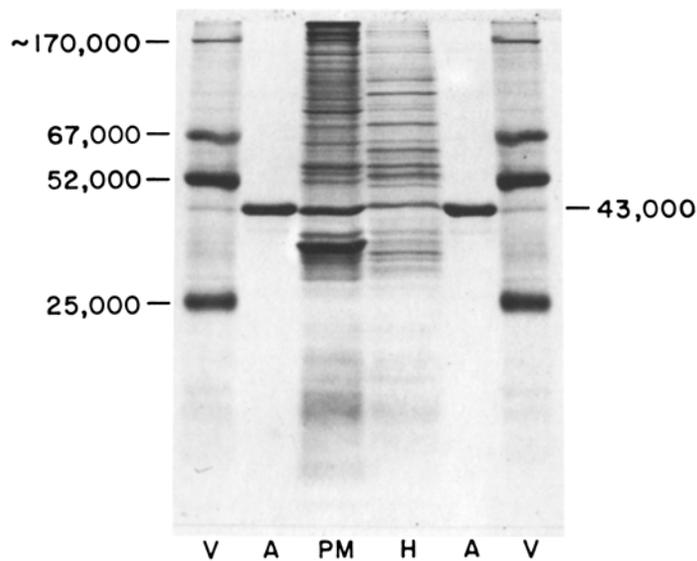


FIGURE 12 Coomassie Blue-stained electrophoretic patterns of homogenate, plasma membrane fraction, and marker proteins, as described in Materials and Methods, using the Tris-glycine discontinuous buffer system. *V* (12.5 μg protein) is vesicular stomatitis virus; *A* (5.75 μg protein) is actin; *PM* (78 μg protein) is the plasma membrane fraction, and *H* (80 μg protein) is the homogenate. The major bands of the virus are L protein (~170,000 mol wt), G protein (67,000 mol wt), N protein (52,000 mol wt), and M protein (25,000 mol wt). Actin shows a mol wt of 43,000. Note that the homogenate shows a number of bands which are not concentrated in the plasma membrane fraction. Likewise, the plasma membrane fraction shows a number of high molecular weight bands which are not prominent in the homogenate. The major bands in the plasma membrane fraction are at 81,000, 43,000, and ~30,000⁴ daltons apparent molecular weight. Densitometry of the negative used to produce this photograph showed the 43,000-dalton band to be 7.8% of the total Coomassie Blue-stained material when the base line was placed at equivalence to the gel in which actin only was electrophoresed. [¹⁴C]-fucose autoradiography of similar gels showed two major glycoproteins, one migrating approximately 1,000 daltons larger than the major (30K) plasma membrane Coomassie Blue-stained band and one 2,000 daltons smaller.

bain-sensitive, Na^+ , K^+ activity (Table I) to a specific activity of $6.50 \pm 1.57 \mu\text{mol}$ of P_i released/mg protein per h. Recovery of the ouabain-sensitive Na^+ , K^+ -ATPase is 12.0%, matching the recovery by counting ghosts in a hemocytometer in the eight experiments used in Table I. Enrichments and recoveries of radioactive fucosyl glycoprotein are paralleled by ouabain-sensitive Na^+ , K^+ -ATPase in the plasma membrane fraction purified by one cycle of rate zonal centrifugation (Table I).

Activity and recovery of Mg^{++} , Na^+ , K^+ -ATPase is also shown, as ouabain-sensitive ATPase activity is the difference between this activity and the activity observed in the presence of 1 mM ouabain. It can thus be determined that the ouabain-sensitive Na^+ , K^+ -ATPase is 37% of the homogenate's total Mg^{++} , Na^+ , K^+ -ATPase, and for the ghost fraction the figure is 83%. Relative

specific activities (and recoveries) are shown as the mean of the ratio in individual experiments. This is not the same estimate of enrichment obtainable by the ratio of means of all the experiments. We have reported the data this way because we regard a relative specific activity to be an independent statistic in each experiment, and hence we can show standard deviations of the means.

Further purification of the ghost fraction by isopycnic banding, at their density of 1.16 g/ml, plus a further 10 mM Tris wash resulted in a

⁴ This particular gel shows the major Coomassie Blue-stained band running at ~30,000 daltons. This was anomalous; several other independent plasma membrane preparations which were run similarly in 10% Tris-glycine buffered gels gave an average of 34,000 daltons.

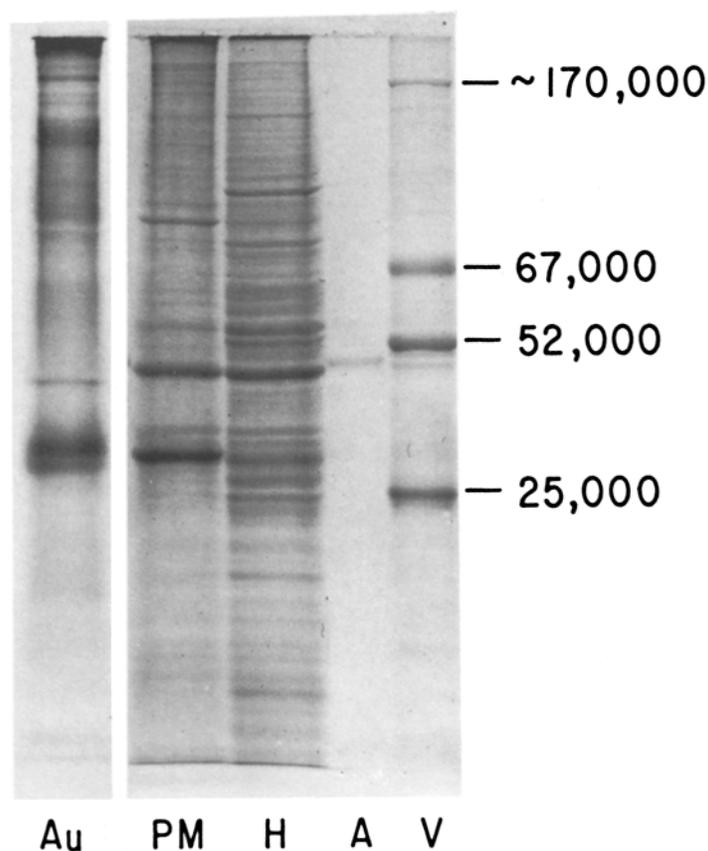


FIGURE 13 Comparison of the positions of the major fucosyl glycoproteins with the Coomassie Blue-stained electrophoretic profile of plasma membranes. Cells were labeled 21 h with $0.03 \mu\text{Ci/ml}$ [^{14}C]fucose. In Materials and Methods are described the preparations of plasma membrane fractions, the SDS-10% polyacrylamide gels, and the autoradiography procedure. The photographed autoradiograph of the plasma membrane profile is labeled *Au*. The Coomassie Blue-stained profiles are of plasma membrane fraction (*PM*), homogenate (*H*), actin (*A*), and vesicular stomatis virus (*V*).

TABLE I
Corecovery and Enrichment of Ouabain-Sensitive Na^+ , K^+ -ATPase, and [^3H]Fucosyl Glycoprotein in Plasma Membrane Ghosts*

	$\text{Mg}^{++}, \ddagger \text{Na}^+$, K^+ -ATPase			Ouabain-sensitive Na^+ , K^+ -ATPase			[^3H]fucosyl glycoprotein		
	Sp act	Relative sp act \ddagger	Recovery	Sp act	Relative sp act \ddagger	Recovery	Sp act	Relative sp act \ddagger	Recovery
	($\mu\text{mol Pi/mg protein/h}$)		(%)	($\mu\text{mol Pi/mg protein/h}$)		(%)	($\text{cpm}/\mu\text{g protein}$)		(%)
Homogenate	1.96 ± 0.93	1	100	0.73 ± 0.18	1	100	19.1 ± 6.4	1	100
Plasma membrane ghosts	7.87 ± 1.70	4.4 ± 1.5	6.4 ± 2.6	6.50 ± 1.57	9.1 ± 2.2	12.0 ± 3.8	151.0 ± 41.5	8.3 ± 1.2	11.9 ± 2.4

* Figures are mean values ($n = 8$) \pm standard deviation.

\ddagger This enzyme activity is the difference between Mg^{++} , Na^+ , K^+ -ATPase activity, and the same activity in the presence of 1 mM ouabain.

\S Mean of the individual experiment relative specific activities.

|| Plasma membrane ghosts purified by one cycle of zonal centrifugation. In four separate experiments, after zonal centrifugation, the relative specific activities of ouabain-sensitive Na^+ , K^+ -ATPase, and radioactive fucosyl glycoprotein were 7.3 ± 1.0 and 7.7 ± 0.5 , respectively, and the recoveries 10.2 ± 0.7 ; 9.6 ± 1.4 . In the same experiments, when the ghosts were then isopycally banded, the relative specific activities of ouabain-sensitive Na^+ , K^+ -ATPase, and radioactive fucosyl glycoprotein were 10.2 ± 2.5 and 9.2 ± 0.6 , respectively, and the recoveries 5.6 ± 2.9 and 4.9 ± 1.7 .

further enrichment of both ouabain-sensitive Na⁺, K⁺-ATPase, and fucosyl glycoprotein, to a mean value of about 10-fold over that at the homogenate (footnote in Table I).

For measuring total enzyme activity recoveries and fucosyl glycoprotein quantity in all cell subfractions including the ghost fraction, the method described in Materials and Methods was modified slightly so as to concentrate the particular fractions. This experiment was performed to establish that enzyme activity is not grossly over-recovered or underrecovered, indicating possible activation or inactivation in the ghost fraction as compared with that of the starting homogenate. Data for the glycoprotein have been included for the sake of comparison only, as quenching of radioactivity is not a problem in the sample sizes used.

After hypotonic disruption, the homogenate was split into a low-speed pellet (500 g, min) that contains the bulk of the nuclei, some plasma membrane ghosts and unruptured cells, and a supernatant fraction that contains cytoplasmic particulates and cytosol as well as most of the ghosts. In general, the complexity of the separations led to some variability in the data obtained. For example, enzyme activity was, on the average, recovered 76% in the first low-speed sedimentation step (Table II) but, including three other experiments, the recovery ranged from 60 to 93%. The low-speed supernatant fraction was further fractionated on a discontinuous sucrose gradient into five bands, subfractions I, II, III, IV,

and V. As previously reported (1, 2), the ghosts recovered in the isolated fraction IV constitute only 10–20% of the original cell number, leaving the other 80–90% of plasma membrane in subfractions I, II, and III, as vesicles and fragments. The total recoveries in all subfractions were 80.2% for protein, 75.0% for enzyme activity, and 77.9% for fucosyl glycoprotein. Enzyme activity and fucosyl glycoprotein are enriched and recovered in the ghost fraction to an almost identical extent (Table II), consistent with the results shown in Table I.

In the two experiments used for Table II, and in several other experiments, the fucosyl glycoprotein recovery in Fraction II was higher than the recovery of ouabain-sensitive Na⁺, K⁺-ATPase activity. This difference may reflect a greater tendency for fucosyl glycoprotein to be located in vesicles apparently forming from breakdown of some plasma membrane ghosts during homogenization. This will require further investigation. For our present purpose, the conclusion from these experiments is unequivocal, namely that the enzyme is neither activated nor inactivated significantly, and thus provides confidence in the accuracy of the observed enrichments of enzyme activity in the ghost preparations (Table I).

DISCUSSION

Microscopy

The observations establish that the ghost fraction utilized in this study for analysis of ouabain-

TABLE II
Recovery of Ouabain-Sensitive Na⁺, K⁺-ATPase, and [³H]Fucosyl Glycoprotein

	Protein		Ouabain-sensitive Na ⁺ , K ⁺ -ATPase				[³ H]fucosyl glycoprotein			
	Total mg	Homogenate (%)	Units (μmol Pi released/h)	Homogenate (%)	Sp act (μmol/mg protein/h)	Relative sp act	CPM (+10 ⁶)	Homogenate (%)	Sp act (cpm/μg protein)	Relative sp act
Homogenate	112.6	100	62.1	100	0.55	1	18.93	100	17.05	1
500 g min pellet	25.3	23.0	10.7	17.2	0.50	0.9	1.75	9.2	9.91	0.6
500 g min supernate	84.2	74.7	36.5	58.8	0.44	0.8	15.62	82.5	20.24	1.2
Total	109.5	97.7	47.2	76.0	—	—	17.37	91.7	—	—
500 g min super										
Subfraction I	47.8	42.4	5.3	8.5 (14.5)	0.11	0.2	2.03	10.7 (13.0)	4.11	0.2
Subfraction II	12.2	10.8	6.6	10.6 (18.1)	0.56	1.0	5.16	27.3 (33.1)	56.72	3.3
Subfraction III	5.7	5.1	6.4	10.3 (17.5)	1.13	2.1	2.37	12.5 (15.2)	39.51	2.3
Subfraction PM	1.7	1.5*	9.1	14.7 (24.9)	5.28	9.6	2.62	13.8 (16.7)	163.07	9.6
Total	67.4	59.8‡ (80.2)	27.4	44.1‡ (75.0)	—	—	12.18	64.3‡ (77.9)	—	—

* In 10 experiments where complete recovery of enzyme and glycoprotein was not determined, recovery of protein in the plasma membrane fraction was $1.27 \pm 0.19\%$ of the homogenate, starting with 4×10^8 cells.

‡ Expressed as a percentage of the original homogenate, figures in parentheses are the percentages of the 500 g min supernate recovered in the various subfractions. The data shown are the average of two consecutive experiments.

sensitive Na^+ , K^+ -ATPase activity, and used in other studies for observing the kinetic behavior of plasma membrane precursors (2, 3, 45) is a highly purified fraction. It is contaminated by fewer than 0.2% nuclei, too little to measure biochemically. Ribosomes, free or bound to endoplasmic reticulum vesicles, are present in the isolated ghosts but in small numbers, accounting for about 3% of the RNA in the initial cells. Although the ghosts appear unvesiculated in light microscopy (Figs. 2 and 3), it is apparent from electron microscopy (Figs. 5-7) that they have large numbers of attached pseudopods and vesicles. Earlier kinetic studies indicate that unidentified internal membranes bearing fucosyl glycoprotein contaminate the ghost fraction to a minor degree (2, 45).

The SDS-polyacrylamide gel electrophoretic analyses show that the ghosts contain two major bands, one of them multiple species with several minor bands. A major band, migrating at 43,000 mol wt, was not glycosylated with fucose, comigrated with purified actin, and probably accounted for the specific staining of the plasma membrane with actin antiserum. Gruenstein, Rich, and Weihing (19) demonstrated the presence of actin (by SDS-gel electrophoresis and by peptide mapping of the eluted protein) in membrane fractions isolated from 3T3 mouse fibroblasts and HeLa cells by a modification of the Brunette and Till Procedure (8) which includes ZnCl_2 during homogenization. In this study, the actin band was not observed to be more concentrated in the plasma membrane fraction than it was in the whole cells, nor was the major protein (apparent mol wt of 34,000) as pronounced in these preparations as in ours. However, a gel electrophoresis profile similar to ours was detected by autoradiography of the [^{35}S]methionine-labeled proteins (10) in HeLa cell plasma membranes prepared by methods described in this and previous papers (1-4). Cohen et al. (11) compared SDS-gel profiles of HeLa plasma membrane proteins in plasma membranes made by our technique (1) and those made by a novel method. Though the gel electrophoresis system was somewhat different from that used in our study, the same major bands can be seen.

One ghost, as purified here, has about 10% of the total cell protein. About 7.5% of this protein is actin or actinlike. These figures compare with an estimate of 1.9% of the total cell protein for red cell ghosts (22) and 8-11% for L cells (18). The higher protein content of the nucleated cell

plasma membranes may reflect a greater plasma membrane complexity than the comparatively simple red cell or that our membrane preparations are more contaminated. It is presently difficult to distinguish between these possibilities. Actinlike polypeptides constitute 4.2% of the red cell ghost protein, with spectrin and myosinlike proteins comprising another 29.8% (40). Gruenstein et al. (19) observed that 4% of HeLa cell plasma membrane protein in cells treated with zinc ions was actin, but did not estimate how much cell protein the plasma membrane ghost contained.

Actinlike and myosinlike proteins are now considered to be present in essentially all eukaryotic cells (36). Electron microscopy shows filaments ~5-7 nm thick under the plasma membranes of many cell types. In 1973, Pollard and Korn (35) showed, by their specific interaction with heavy meromyosin to form polarized arrowhead structures, and by other characteristics, that the filaments associated with the inner surfaces of plasma membranes isolated from *Acanthamoeba* contained actin or actinlike material. The fibrillar material seen in our electron micrographs probably includes the actinlike protein, and its close association with the ghosts may reflect important physiological processes attributed to such proteins in nonmuscle cells (reviewed by Nicolson, 30).

Painter, Sheetz, and Singer (31) demonstrated that relatively intact cells which were present in homogenates of ZnCl_2 -treated VI38 cells were permeable to ferritin-conjugated antibody against myosin (or myosinlike material). The conjugate was associated with the cytoplasmic surfaces of the plasma membranes.

Previous studies (4) had shown that fucosyl glycoprotein corecovered fairly well with the ghosts. The present study demonstrates that radioactive fucosyl glycoprotein copurifies and enriches in purified ghosts to the same extent as ouabain-sensitive Na^+ , K^+ -ATPase activity (Tables I, II). Fucose-labeled macromolecules in HeLa cells are mostly glycoprotein (2); a similar situation has been reported for BHK 21 cells, where it was established that ganglioside sialic acid (38) was as good a marker for plasma membrane as Na^+ , K^+ -ATPase. In another study, fucosyl glycoprotein (17) was suggested as being a good plasma membrane marker, although coenrichment and corecovery studies were not performed. Both ^{125}I surface-labeled proteins and fucosyl glycoproteins were observed to copurify to approximately the same extent in the same preparations of plasma

membranes prepared from chick embryo fibroblasts, an average of 14.5-fold and 12-fold, respectively (29). Similarly, Smith and Crittenden (39) reported a coenrichment of radioactive fucosyl glycoprotein and cytidine 5'-triphosphatase (CTPase) in a plasma membrane fraction prepared from cultured chick embryo fibroblasts. Cohen et al. (11), using our techniques (4) and also those of Johnsen et al. (20), reported an enrichment of ouabain-sensitive Na⁺-K⁺-ATPase in purified HeLa plasma membranes very similar to that reported here. ¹²⁵I-labeled wheat germ agglutinin but not ATPase surprisingly was enriched to a much higher extent in the novel (11) bead preparative method. From a kinetic study of the synthesis and fate of fucosyl glycoproteins in HeLa S₃ cells, Yurchenco and Atkinson (45) have estimated that about 96% of the total cell fucosyl glycoprotein is bound to the plasma membrane and 2% or less is in an internal pool precursor to the plasma membrane. However, in HTC cells (a rat hepatoma line grown in suspension culture), approximately 50% of the fucosyl glycoprotein is not associated with the plasma membrane fraction (41), and a significant portion of this pool is precursor to plasma membrane glycoprotein (13). The intracellular sites of synthesis and possible functional roles of the major nonglycosylated and glycosylated proteins in different cell types require further investigation.

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