A NEW RAPID METHOD FOR THE ISOLATION OF
SURFACE MEMBRANES FROM TISSUE CULTURE CELLS

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A number of methods for the isolation of the sur-
face membrane of animal cells have been described
(1–5). All these methods involve rupture of the
cells and separation of the surface membranes from
the resulting homogenate by physical techniques.
The identity and purity of the isolated product
have been judged by morphological criteria, and
the enrichment of enzymatic activities associated
with the surface membrane. Warren and his co-
workers (3) stabilized the cell surface membrane
prior to rupture to prevent fragmentation of the
membrane. All of these methods are lengthy and
result in a low yield of the final membrane fraction.

This report describes a rapid and simple method
for the isolation of a highly enriched surface mem-
brane fraction from monolayer and multilayer
tissue culture cells. Phase-contrast-and electron
microscopy of the membrane fraction reveals very
little contamination with other cell organelles. The
method also allows for simple microscopic or elec-
tron microscopic examination of the remaining
cellular material after removal of membrane.

METHODS

Tissue Culture

The 3T6 mouse embryo cells were obtained from
Dr. Howard Green. The cells were grown in poly-
styrene tissue culture flasks[1] (75 cm²) in Dulbecco-
Vogt's modification of Eagle's medium containing

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[1] Falcon Plastics, Los Angeles, Calif.

10% calf serum under 10% CO_2. The medium was changed twice a week and subcultures were made by trypsinization of confluent cultures. Membranes were isolated from confluent cultures consisting of multilayers two to five cells thick (2–6 $\times$ 10⁶ cells per flask).

Human synovial cells were grown in polystyrene Petri dishes from explants of synovium obtained at open biopsy. The primary cultures were subcultured in plastic flasks in Dulbecco-Vogt's modification of Eagle's medium containing 10% calf serum. The medium was changed twice weekly. Membranes were isolated from confluent monolayer cultures (0.5–1.5 $\times$ 10⁶ cells per flask) between the third and fifth subcultures.

Membrane Isolation

The medium was decanted from the culture flask and the cell layer washed gently three times with 10-ml portions of 0.16 M NaCl containing 0.01% $CaCl_2$ prewarmed to 37°C. The cells were then covered for 10 min with a solution consisting of 4 volumes 0.001 M $ZnCl_2$ to 1 volume dimethyl sulf-

oxide (DMSO) at room temperature. This solution was decanted and the cells were immediately covered with 20 ml of saturated fluorescein mercuric acetate (FMA) in 0.02 M Tris buffer, pH 8.1, prepared according to the method of Warren et al. (3). The flask was placed flat in an ice bath and shaken on a rotating platform at approximately 120 rpm. A drop of the FMA was removed and inspected by phase-contrast microscopy at 30 and 45 min. If, in addition to increasing numbers of cell membrane ghosts, some whole cells or nuclei were seen, the shaking was stopped at this point. If no whole cells or nuclei were seen, the shaking was stopped after 60 min. The FMA containing a suspension of membranes was decanted into a 30 ml conical centrifuge tube. If portions of the cell layer growth were torn loose during the period of shaking, they could be removed by filtration through three layers of cheesecloth. The FMA solution containing a suspension of cell membranes was centrifuged in a swinging bucket centrifuge at 600 g for 10 min at 4°C. The supernatant which was clear on phase-contrast microscopic examination was aspirated and discarded. The orange
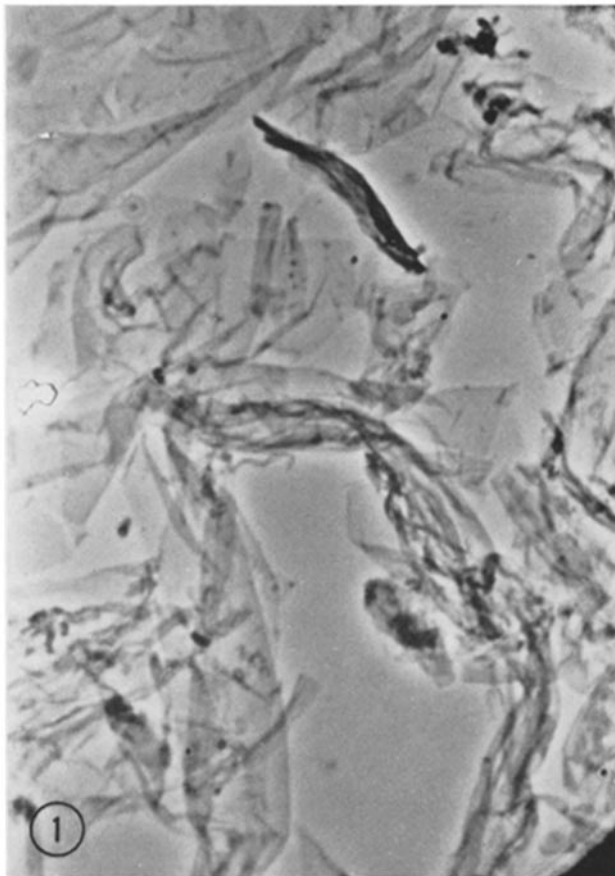

FIGURE 1 Photomicrograph of a typical final membrane preparation from a culture of 3T6 cells, showing flattened folded membranes. The straight smooth margins of unfolded membranes are a characteristic feature of the membranes prepared by this method. There is very little granular material. $\times$ 870.

pellet was resuspended in 20 ml of cold 1 mM NaHCO₃ and again centrifuged at 600 *g* for 10 min. The washing procedure was repeated three times with 1 mM NaHCO₃. The final pellet was suspended in 1-2 ml of 1 mM NaHCO₃.

Electron Microscopy

A portion of the final pellet of membranes was suspended in cold 1% osmium tetroxide in Millonig's buffer for 30 min. The membranes were then centrifuged, and 1 ml of melted 1% agar at 50°C was added to the pellet which was prewarmed to 40°C in a water bath. The membranes were agitated into suspension and then recentrifuged at 1200 *g* for 10 min at 40°C in order to keep the agar in a liquid state. The centrifuge tube was then chilled in an ice bath and the gelled agar was removed from the centrifuge tube as a block with the aid of a wooden applicator stick. 1 mm cubes of agar containing membranes were cut out and dehydrated through alcohols. The last 30 min soak in absolute alcohol contained 2% uranyl acetate. The blocks were then cleared in propylene oxide and embedded in araldite. Sections were cut at different levels in the blocks with a diamond knife, stained with lead citrate, and examined with a Siemens Elmiskop I electron microscope (Siemens America, Inc., New York).

The cell layer remaining after the removal of the FMA suspension was fixed *in situ* with 1% osmium tetroxide for 30 min. The cell layer was then scraped into suspension by using a rubber policeman, and was encased with agar as described above. The agar blocks were dehydrated, embedded in araldite, sectioned, and examined in the same manner as the membrane fractions.

Protein Determination

Portions of membranes and the cell layer remaining after removal of membranes were centrifuged and the pellets were dissolved in 1 N NaOH. Protein was determined in these solutions by the method of Lowry (6). Solutions of bovine serum albumin containing 0.01% FMA were used as standards.

RESULTS

Phase-contrast microscopy of the final membrane suspension from 3T6 and synovial cells revealed a remarkably uniform appearance of large membrane fragments. The membranes had the appearance of segments of cellophane that were randomly folded and curled. The edges of the membrane fragments were smooth and straight. Occasionally the membranes appeared to adhere to each other, forming long ropelike structures. Only a rare nucleus or whole cell was seen. No cytoplasmic structures were recognizable (Fig. 1).

Phase-contrast microscopy of the cell layer *in situ* after membrane isolation revealed many cells without a distinct surface membrane. Nuclei with easily visible nucleoli and nuclear membranes, as well as cytoplasmic vacuoles and granules, were readily apparent (Fig. 2).

Electron microscopy of the final membrane pellets revealed large numbers of highly folded and tortuous membranes in which a unit membrane substructure could be seen (Fig. 3). A thin layer of amorphous and filamentous material was regularly seen attached to the membranes. Granules morphologically similar to ribosomes and occasional clusters resembling polyribosomes were noted attached to the membranes. In none of the many sections examined were nuclear or recognizable cytoplasmic organelles, other than rare small vesicles, seen.

Examination of the cell layers, after membrane removal, in the electron microscope revealed two easily distinguished cell populations. One group of cells had remarkably well preserved cytoplasmic fine structure. Typical cisternae of rough endoplasmic reticulum, portions of the Golgi apparatus, mitochondria, polyribosomes, and multivesicular bodies were readily seen. These cells were surrounded only in part by a plasma membrane. However, large segments of these cells appeared to lack a clearly defined cell membrane (Fig. 4). The proportion of such areas appeared much too great to be accounted for by tangential sectioning alone. The other group of cells was characterized by a very densely granular cytoplasm in which only occasional vesicular structures and swollen mitochondria were recognizable. The nuclei of these cells appeared less dense and lacked typical dense chromatin patterns. The nuclear membranes were, however, usually discernible. Many of these dense cells appeared to lack a plasma membrane over the major portion of the cell circumference. Identical results were found for both the monolayer synovial cells and the multilayer 3T6 cells.

Protein recovery in the final 3T6 cell membrane fraction was 8% of the total obtained by combining the cell layer remaining after membrane removal and cell membrane fractions (mean of six experiments, SD 5.1%).

DISCUSSION

The procedure for the isolation of surface membranes from tissue culture cells described here is a modification of the methods originally reported by
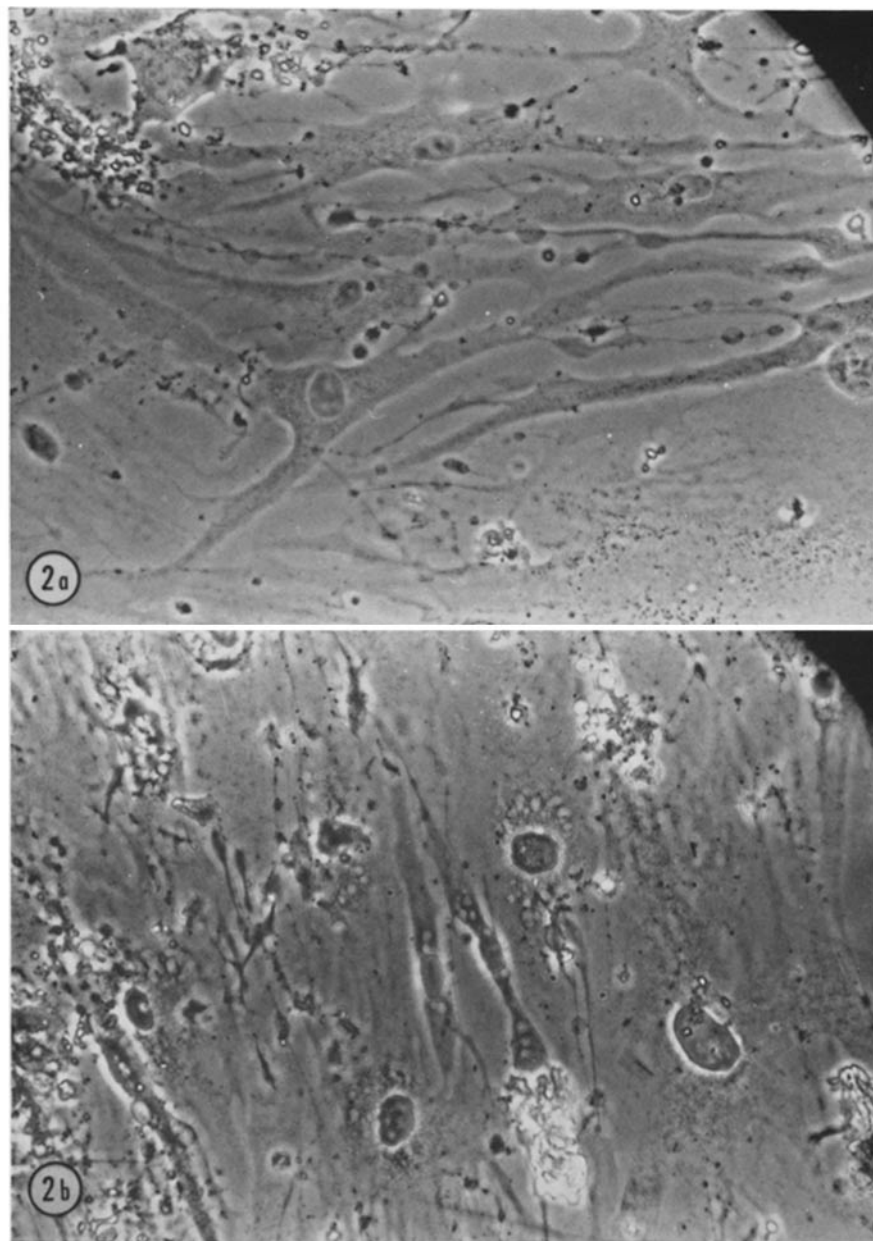
FIGURE 2 Synovial cells photographed at different stages of membrane preparation. (a) Cells immediately after the addition of $ZnCl_2$ and DMSO. The distinct cell margins are clearly seen. (b) The same cell culture after shaking in FMA for 1 hr. Many of the cell margins appear quite indistinct or unrecognizable. Other changes in the cells include swelling of the nuclei and vacuole formation in the cytoplasm. × 220.

Warren et al. (3). The cell membrane is stabilized and hypotonically stretched away from the cell body. The mechanism by which zinc and FMA act to strengthen the cell membrane is not well understood. Zinc ions are capable of reacting with a wide variety of ligands in the cell and are able to precipitate proteins from salt solutions. FMA is a potent sulfhydryl-blocking reagent and may act
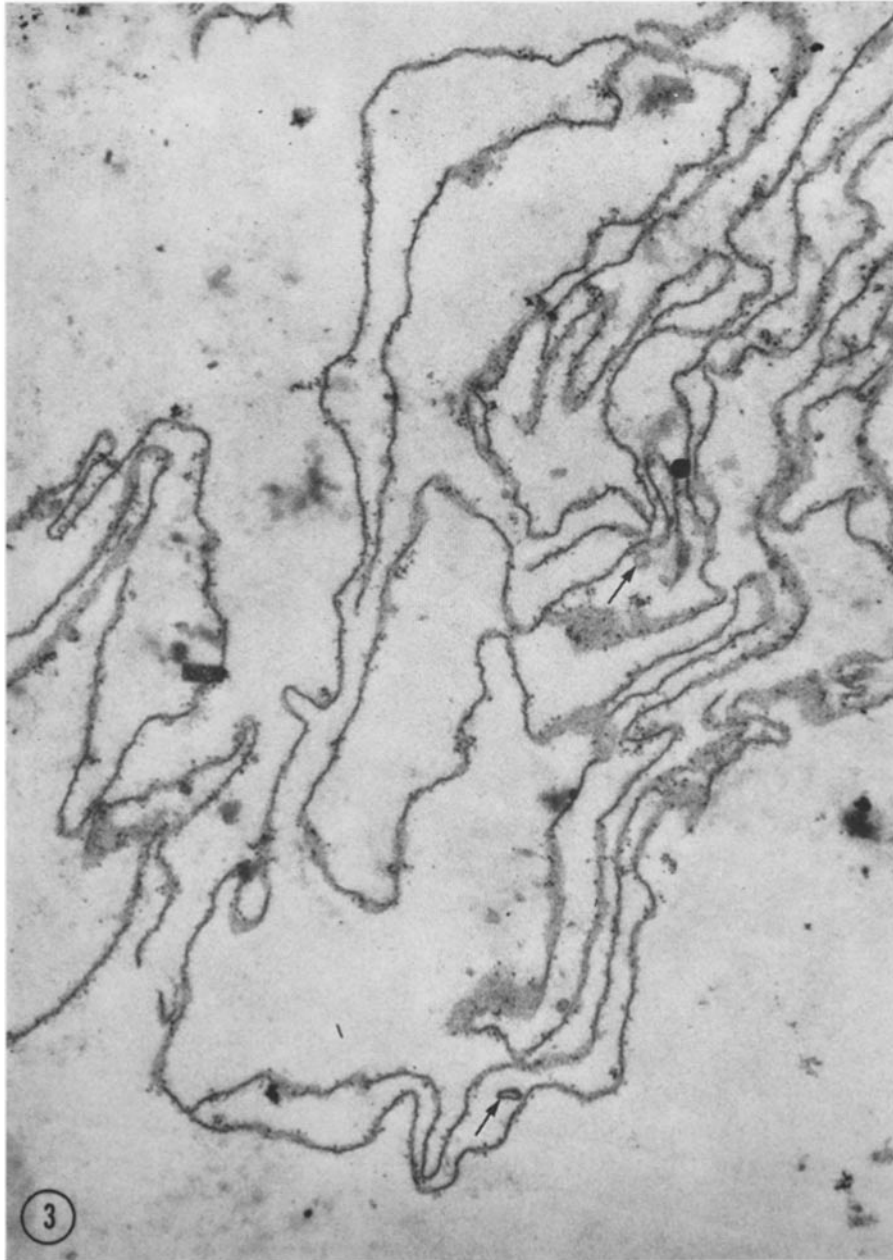
FIGURE 3 Electron micrograph of a membrane preparation from a culture of 3T6 cells. The long segments of membranes with attached granules are apparent. Occasional vesicles closely associated with the membranes are also seen (arrows). $\times$ 14,000.

through this property. The membranes are then shaken from the body of the cell which remains adherent to the culture vessel.

A number of advantages of this procedure should be noted. The method is simple and rapid. A membrane preparation is obtained in less than 2 hr and does not require the use of complex equipment. The risk of losses during transfer and bacterial contamination are minimized.
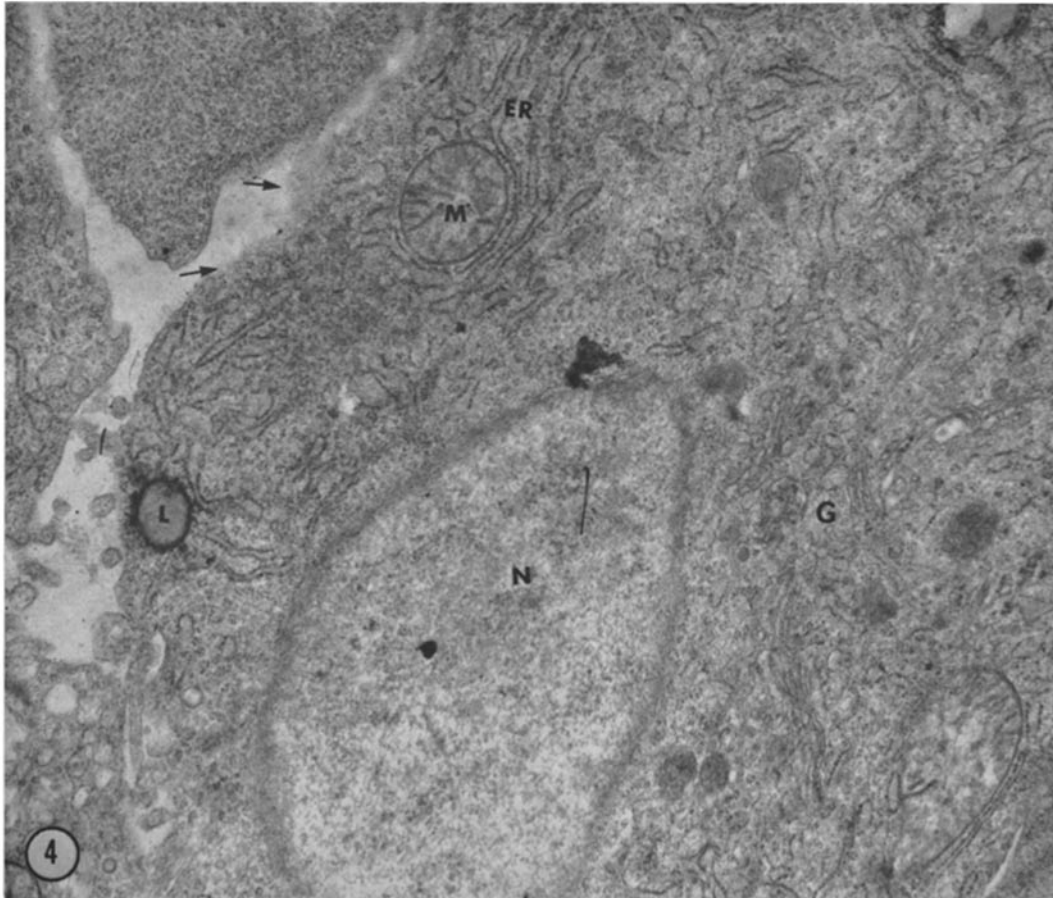
The procedure appears to result in a highly

FIGURE 4   Electron micrograph of 3T6 cells after isolation of the cell membrane fraction. The remarkable preservation of cellular ultrastructure is seen. Areas of the Golgi apparatus (G), endoplasmic reticulum (ER), mitochondria (M), and lipid vacuoles (L) as well as the nucleus (N) are seen. However, a portion of the cell membrane appears missing (arrows). $\times$ 12,000.

homogeneous membrane preparation, as judged by morphological criteria. RNA is found in plasma membranes prepared by other methods and may be in attached ribosomes as a regular feature of this organelle. The purity of the membranes is further substantiated by electron microscopy of the cells remaining after membrane separation, which shows that most of the cellular organelles are intact aside from the surface membrane.

An additional advantage of this procedure is that the use of trypsin with its effects on the membrane is avoided. Trypsinization has been used previously to suspend tissue culture cells prior to rupturing, resulting in alterations of the cell surface. Cook et al. have reported changes in the electrophoretic mobility of Erhlich ascites carcinoma

cells after treatment with trypsin (7). Trypsin also liberates a sialomucopeptide from human erythrocytes (8).

We are currently evaluating our membranes for residual enzymatic and antigenic activity. It is probable that very little biological activity remains in membranes isolated in $ZnCl_2$ and FMA. However, Glick and Warren (9) report protein synthesis in cell membranes isolated after stabilization with zinc chloride.

It is evident that our surface membranes represent only a selected portion of the entire cell surface, since some of the membranes remain adherent to the culture flask; perhaps these membranes represent functional areas of the surface in contact with the culture medium. Other methods for the

isolation of surface membranes may also provide only a limited sampling of the entire surface area. Wallach (10) has estimated that hepatic cell membranes isolated by the method of Emmelot represent at most 14% of the entire surface membrane. That other methods are also selective rather than random is indicated by the varying enhancement of specific activity for different enzymes believed to be associated with the surface membrane (5). Where whole ghosts have been isolated, it has been found that some of the cells in the suspension are resistant to hypotonic swelling. Therefore, this technique may be subject to a degree of selectivity as well.

It is hoped that this rapid method for obtaining cell surface membranes will provide a useful means for studying the structure and chemistry of this vital cell organelle.

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