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CHARACTERIZATION OF THE MEMBRANE FRACTION ISOLATED BY THE FLUORESCEIN MERCURIC ACETATE TECHNIQUE OF BARLAND AND SCHROEDER

JOSEPH A.McCLURE [a], WERNER FISCHLSCHWEIGER [b] and KENNETH D. NOONAN [a,*]

[a] *Department of Biochemistry and Molecular Biology and* [b] *Department of Basic Dental Sciences, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610 (U.S.A.)*

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Summary

Using scanning electron microscopy we have demonstrated that the membrane fraction isolated by the fluorescein mercuric acetate technique of Barland and Schroeder (Barland, P. and Schroeder, E.A. (1975) J. Cell Biol. 45, 662—668) represents a topologically distinct membrane which circumscribes the cell nucleus. Our data suggest that not all the cells within a non-synchronized cell population release a membrane fraction after treatment according to the technique of Barland and Schroeder, but rather that the efficiency of membrane release achieved using this preparative technique is dependent on the morphology of individual cells.

Our work has also demonstrated that the peptide composition of the membrane fraction isolated by the technique of Barland and Schroeder differs from the peptide composition of the plasma membrane-enriched fraction isolated by the technique of Brunette and Till (Brunette, D.M. and Till, J.E. (1971) J. Membrane Biol. 5, 215—224). This difference in peptide composition is particularly noticeable among the higher molecular weight proteins, glycoproteins and iodineateable membrane components.

The data which we have accumulated suggest that the compositional differences noted between the two membrane isolates do not result from differential extraction of membrane components during the $ZnCl_2$-fluorescein mercuric acetate treatments required in the isolation technique originally described by Barland and Schroeder. However, our data do clearly demonstrate that the membrane isolation technique of Barland and Schroeder cannot be used to study the general composition of the plasma membrane.

* To whom correspondence should be addressed.

Introduction

Over the last decade many different techniques have been developed for isolating the plasma membrane from cells maintained in vitro. Most of these techniques have required the investigator to remove the cells from the substratum prior to isolating the plasma membrane. Concern has been expressed that removal of the cells from the substratum might alter the composition of the plasma membrane [1]. In order to avoid the artifacts which might be introduced into the characterization of the plasma membrane as a result of the removal of the cells from the substratum, a number of isolation procedures have been described which reportedly permit the isolation of defined areas of the plasma membrane from cells still attached to the substratum. These membrane isolation procedures have included formaldehyde [2] and glycerol [3] blistering of cells with the subsequent isolation of membrane vesicles as well as isolation of membranes from ingested latex spheres [4,5]. One relatively popular membrane isolation technique which does not require removal of the cells from the substratum is the technique first described by Barland and Schroeder [6]. This technique reportedly permits the isolation of the upper surface of sustratum-adherent cells.

In this paper we present morphological data which define the area of the plasma membrane isolated by the technique of Barland and Schroeder [6]. We also characterize the peptide and glycopeptide composition of this membrane fraction and compare it to the composition of the plasma membrane fraction isolated from the same cells via the technique of Brunette and Till [7].

Materials and Methods

Materials. All reagents used in electrophoretic analysis of plasma membrane peptides were purchased from Bio-Rad Laboratories (Rockville centre, N.Y.). Tissue culture media and sera were obtained from Grand Island Biological Co., (Grand Island, N.Y.). Tissue culture flasks and dishes were purchased from Corning Glass Works (Corning, N.Y.). All radioisotopes (except ^{125}I) and scintillation cocktail were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). ^{125}I was purchased from Schwarz/Mann Division Becton, Dickinson and Co. (Orangeburg, N.Y.). Lactoperoxidase was obtained from Calbiochem (La Jolla, Calif.). Concanavalin A was obtained from Miles-Yeda (Miles Laboratories, Inc., Elkhart, Indiana) and then affinity purified on Sephadex G-100. Dextran 500 was obtained from Pharmacia (Piscataway, N.J.). All reagents for electron microscopy were purchased from Polysciences, Inc. (Warrington, Pa.). Fluorescein mercuric acetate was obtained from Sigma Chemical Co. (Stl. Louis, Mo.). All other laboratory reagents were purchased from Scientific products (Ocala, Florida).

Maintenance of the cell line. The cell line used throughout this work was a spontaneous transformant of a Balb/c 3T3 mouse embryo cell line (clone A-31). This spontaneous transformant was isolated in our laboratory and is referred to as sptr 3T3 throughout the manuscript. The cell line was maintained in Dulbecco modified Eagle's medium supplemented with 10% (v/v) calf serum. Cells were passaged every two to three days at approximately 80% confluency.

The cells were routinely tested for PPLO contamination by both autoradiography [8] and nutrient agar [9] and were found to be free of contamination. Under the growth conditions used the spontaneous transformant (sptr 3T3) grew to a final cell density of approximately $1 \cdot 10^5$ cells/cm^2. In the experiments reported throughout this paper, cell cultures were used for membrane isolation when the cell density reached approximately $8 \cdot 10^4$ cells/cm^2.

Membrane isolation. 1. Aqueous two-phase polymer technique of Brunette and Till [7]: sptr 3T3 cells were removed from the tissue culture dish by washing the plates three times with calcium, magnesium-free phosphate-buffered saline (pH 7.2) containing 0.2% EDTA and 1 g/l glucose and then incubating the cells in calcium, magnesium-free phosphate-buffered saline containing EDTA and glucose, for 15 min at 37°C. We found that the addition of glucose to calcium, magnesium-free phosphate-buffered saline-EDTA increased the rate at which the sptr 3T3 cells were released from the substratum and improved the relative viability of the cells released. Following release from the substratum the cells were washed three times with phosphate-buffered saline (pH 7.2) and then a membrane fraction enriched in plasma membrane was isolated via the aqueous two-phase polymer system described by Brunette and Till [7]. Membranes were banded at least three times in aqueous polymer and then washed with phosphate-buffered saline to remove excess polymer. In those experiments where enzyme activity was monitored, the final membrane fraction was washed with 1 mM Tris-HCl/10 mM EDTA (pH 7.5) instead of phosphate-buffered saline to remove excess zinc [10].

2. Fluorescein mercuric acid technique of Barland and Schroeder [6]: sptr 3T3 cells were grown to 80% confluency. The medium was decanted and the cells washed 5 times with either phosphate-buffered saline or 0.16 M NaCl + 0.01% $CaCl_2$ (37°C). The cells were then 'tanned' for 10 min (room temperature) with 1 mM $ZnCl_2$ in dimethylsulfoxide (Me_2SO) (4 : 1). Following the tanning process the $ZnCl_2/Me_2SO$ was decanted and cold, saturated fluorescein mercuric acetate (approx. $2.2 \cdot 10^{-3}$ M) in 0.02 M Tris-HCl (pH 8.1) was added. The cells were placed on ice and shaken (still on ice) for 40 min on a rotary shaker set at 120 rev./min. Material released into the fluorescein mercuric acetate/Tris solution was decanted and pelleted at $600 \times g$ for 10 min. The resulting pellet was washed twice with either 1 mM $NaHCO_3$ or phosphate-buffered saline and then resuspended in 25% (w/v) sucrose. This fraction was layered onto a 50% (w/v) sucrose cushion and centrifuged for 1 h at $200 \times g$. Under these conditions the membranes pelleted. The pellet was collected and washed twice more with phosphate-buffered saline. Attempts to isolate membrane without the $ZnCl_2/Me_2SO$ 'tanning' step or by 'tanning' with $ZnCl_2$ in the absence of Me_2SO all failed. Similarly, membranes could not be isolated from cells which were treated with $ZnCl_2/Me_2SO$, but not fluorescein mercuric acetate.

Using the conditions reported here the Barland and Schroeder [6] technique was equally effective in isolating membrane from cells at densities ranging from $1 \cdot 10^4$ cells/cm^2 to $1 \cdot 10^5$ cells/cm^2. At higher cell densities the relative contamination of the membrane fraction with nuclei or whole cells increased dramatically while at lower cell densities the membrane yield was unacceptably low.

Electron microscopy. Membrane samples were prepared for transmission electron microscopy by fixation in 2% glutaraldehyde (1 h, 4°C) followed by dehydration through successively increasing concentrations of ethanol. Fixed samples were embedded in Spurr's low viscosity medium [11] and sectioned. Silver sections were post-stained with uranyl acetate and examined with a Siemens Elmiskop 101 at 80 kV.

For scanning electron microscopy, cells were grown on 10 × 60 mm glass coverslips until approximately 80% confluent. The cells were washed three times with phosphate-buffered saline and then processed through the various steps described above for the Barland and Schroeder isolation procedure. Samples were fixed overnight in cacodylate buffered (pH 7.4) glutaraldehyde (2.5%) and then dehydrated through successively increasing concentrations of ethanol. Following dehydration the cells, still attached to coverslips, were critical-point dried, mounted and coated with gold and palladium in a 'Hummer II' coating apparatus. The samples were examined in a Zeiss Novascan scanning electron microscope.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Membrane peptides and glycopeptides were separated using the discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel system designed by Laemmli [12]. The slab gels used throughout this work consisted of a running gel with a linear gradient of 7.5—12.5% acrylamide and a 5.6% stacking gel. The acrylamide to bisacrylamide ratio was maintained at 37.5 : 1 in both the stacking and running gel. The gradient gel efficiently separated membrane components with molecular weights approx. 300 000 and 15 000. Following electrophoresis the slab gels were immediately fixed in 10% trichloroacetic acid, stained with Coomassie Brilliant Blue and destained according to the technique of Weber and Osborn [13].

Labeling cellular components. A. Metabolic labeling: Proteins were metabolically labeled by maintaining cells for 72 h in medium containing 2.5 μCi/ml L-[4,5-^{3}H]leucine or 5.0 μCi/ml L-[G-^{3}H]proline. Glycoproteins were metabolically labeled by maintaining cells in medium supplemented with 2.5 μCi/ml D-[1-^{3}H]glucosamine hydrochloride or 1.0 μCi/ml L-[G-^{3}H]fucose. Phospholipids were labeled by maintaining the cells for 72 h in medium containing 2.5 μCi/ml [*me*-^{3}H]choline chloride or 2.5 μCi/ml ortho[^{32}P]phosphate.

B. Surface labeling: In all of our attempts to preferentially label surface components of the plasma membrane, cells were radiolabeled while still attached to the substratum, although removal of the cells from the plate prior to labeling did not markedly influence the pattern of labeling. Prior to iodination the sptr 3T3 cells were washed three times with phosphate-buffered saline and then labeled with 400 μCi/ml ^{125}I according to the lactoperoxidase technique of Philips and Morrison [14]. Iodinated membrane components were separated on SDS polyacrylamide gels and then displayed autoradiographically.

C. Concanavalin A-binding glycopeptides: Following electrophoretic separation of membrane peptides and glycopeptides, the concanavalin A-binding glycopeptides were labeled in the acrylamide slab gels with affinity purified ^{125}I-labeled concanavalin A as described by Burridge [15]. The concanavalin A-binding glycoproteins were displayed by preparing an autoradiogram of the dried gel.

Iodination of calf serum. 50 ml of calf serum were incubated with 500 μCi ^{125}I and labeled according to the lactoperoxidase-catalyzed technique of Phillips and Morrison [14]. Following iodination the serum was dialyzed for 48 h (4°C) against 2 l of phosphate-buffered saline with changes in the dialysate being made every 12 h. After 48 h of dialysis the calf serum reached a constant specific activity of $4.3 \cdot 10^4$ cpm/mg protein ($6.5 \cdot 10^6$ cpm/ml serum). The labeled serum supported cell growth as effectively as unlabeled serum.

Fluorography. The distribution of ^{3}H-labeled cellular components separated on SDS polyacrylamide slab gels was displayed via the fluorographic technique of Bonner and Laskey [16].

RNA, DNA and protein determinations. Due to the interference of the brilliant orange fluorescein mercuric acetate with both the diphenylamine [17] and acid orcinol [18] assays it was necessary to combine these assays with [^{3}H]thymidine or [^{3}H]uridine labeling of the cells in order to determine the relative contamination of the various membrane fractions with DNA or RNA. To determine DNA contamination, cells were grown for 72 h in the presence of 2.5 μCi/ml [*me*-^{3}H]thymidine. Aliquots of the cells were lysed and the specific activity of the DNA determined via the diphenylamine assay [17]. The specific activity of DNA was then used to determine the relative amount of DNA associated with the various membrane fractions. The relative contamination of the membrane fractions with RNA was determined in the same manner except cells were grown for 72 h in [5,6-^{3}H]uridine and the specific activity determined using the acid orcinol [18] technique. Protein concentration was determined via the procedure of Lowry et al. [19].

Phospholipid determination. The phospholipid content of the membranes isolated via the Barland and Schroeder technique [6] and the Brunette and Till technique [7] was determined from a chloroform/methanol extract of the two membrane fractions. Lipid phosphorus was determined via the method of Chen et al. [20] and a factor of 25 used to convert μg phosphorus to μg phospholipid.

Enzyme assays. ($Na^+ + K^+$)-ATPase was measured according to the technique described by Brunette and Till [7]. Inorganic phosphate release was measured via a modification of the Fiske-Subbarow technique [20].

Results

As shown in Fig. 1A, and as has been shown by a number of other workers, pre-treatment of sptr 3T3 cells with $ZnCl_2$/Me_2SO-fluorescein mercuric acetate followed by agitation on a rotary shaker releases membranous material from cells attached to the substratum. During the release of this material from the cells most of the cell remains associated with the substratum (see scanning electron micrographs, in particular Fig. 2F). Approximately 4% of the total cell protein is isolated in the membranous fraction released from the cells.

As can be seen in Fig. 1A the material isolated by our modification of Barland and Schroeder's [6] original technique represents a membrane fraction free of any obvious contamination with other cellular organelles. The only apparent contaminant of the membrane fraction are particles adhering to one side of the membrane. These particles may represent some cytoplasmic material

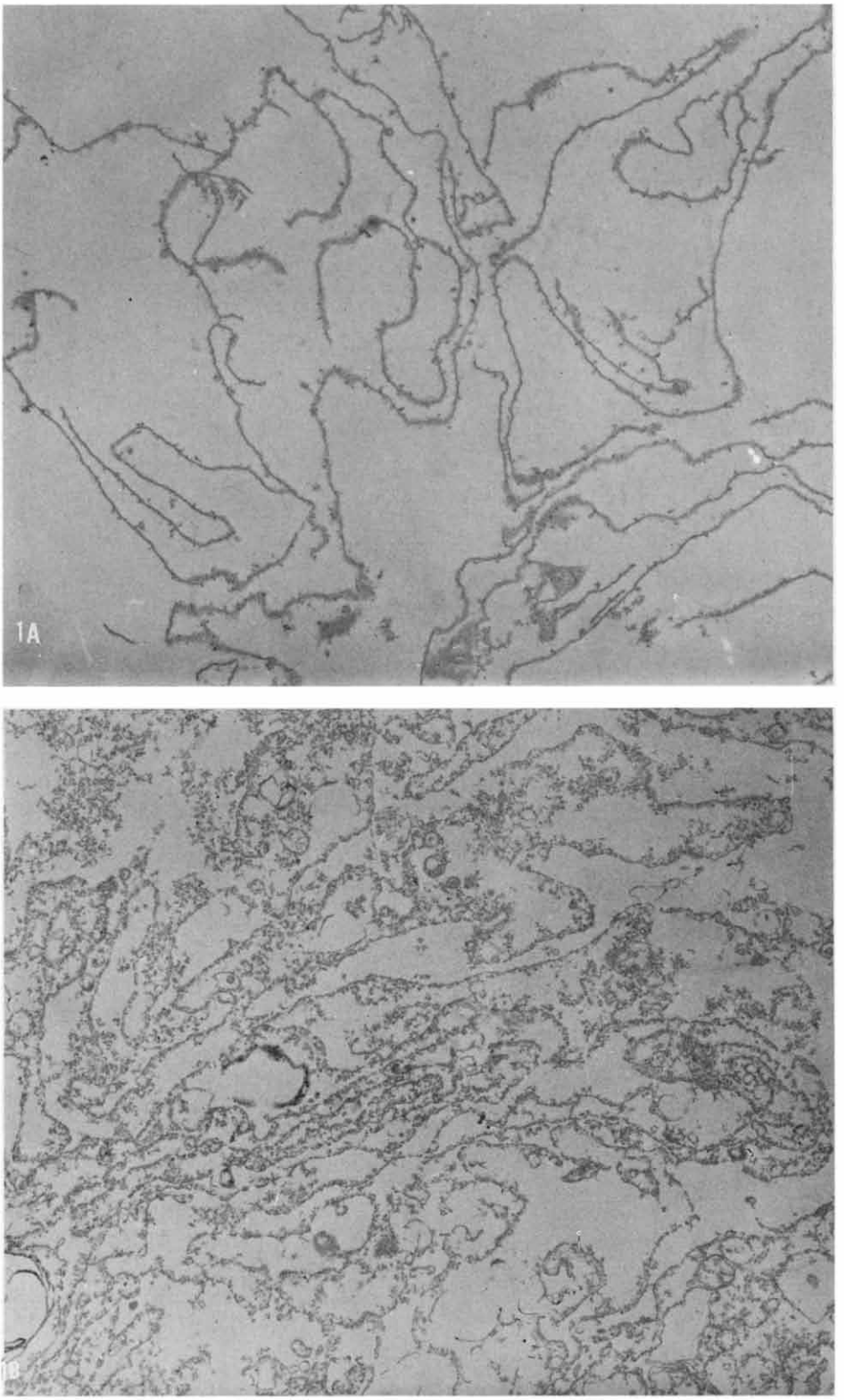


Fig. 1. A. Transmission electron micrograph of membranes isolated according to Barland and Schroeder and then fixed as described in Materials and Methods (×4800). B. Transmission electron micrograph of membranes isolated according to Brunette and Till and then prepared as described in Materials and Methods (×5000).

TABLE I

RELATIVE PURITY OF MEMBRANE ISOLATES

$(Na^+ + K^+)$-activated ATPase was assayed according to the technique outlined by Brunette and Till [7]. Inorganic phosphate release was measured according to the method of Chen et al. [20]. DNA was determined in [^{3}H]thymidine-labeled whole cell homogenates via the technique of Burton [17]. The contamination of the membrane fractions with DNA was determined from the CPM [^{3}H]thymidine associated with the respective membrane fractions and then working back to the relative contamination using the pre-determined specific activity of DNA. RNA was determined in [^{3}H]uridine-labeled whole cell homogenates by the acid-orcinol procedure [18]. RNA contamination of the membrane fractions was determined in the same way as the DNA contamination using the specific activity of RNA. N.D., not determined because the cells had been treated with $ZnCl_2$/Me_2SO-fluorescein mercuric acetate which destroys enzyme activity.

Membrane fraction isolated according to	$(Na^+ + K^+)$-ATPase (μmol P_i/mg protein per h)	DNA (μg/mg protein)	RNA (μg/mg protein)
Brunette and Till	7.65	25.0	150.6
Barland and Schroeder	N.D.	2.9	2.8
Homogenate	0.51	117.3	175.3

(possibly ribosomes) adhering to the underside of the plasma membrane. The data in Table I suggest that the DNA and RNA contamination of the membrane fraction isolated by the Barland and Schroeder [6] technique is insignificant.

We have also isolated a membranous fraction from cells which have been removed from the substratum by repeated washes with calcium, magnesium-free phosphate-buffered saline containing glucose, lysed in 1 mM $ZnCl_2$ and subsequently banded in the aqueous polymer system described by Brunette and Till [7]. This procedure would be expected to isolate the entire plasma membrane since it does not depend on the adhesion of one compartment of the membrane surface to the substratum during the isolation. The membranous fraction derived by this procedure is shown in Fig. 1B. This membranous fraction appears free of gross contamination with cellular organelles although, as has been reported by others [7], more cytoplasmic ground substance adheres to this fraction than to the fraction isolated according to Barland and Schroeder. Approximately 6% of the total cell protein is isolated in the membranous fraction derived from the aqueous polymer system of Brunette and Till.

The data in Table I indicate an approximately 15-fold increase in the specific activity of $(Na^+ + K^+)$-ATPase in the membranous fraction isolated according to Brunette and Till relative to the homogenate, suggesting that the fraction isolated is predominantly plasma membrane. Table I suggests that there is limited contamination of this fraction with DNA; however the contamination with RNA is relatively high. The inclusion of RNA in the membrane-enriched fraction could result from contamination of the fraction with endoplasmic reticulum or might represent cytoplasmic RNA (e.g. free ribosomes). Since the specific activities of the enzyme markers frequently used to distinguish endoplasmic reticulum from plasma membrane cannot be determined in the sptr 3T3 cells due to the inability to isolate pure endoplasmic reticulum, the source of the RNA contamination of the membrane fraction cannot be unequivocally determined.

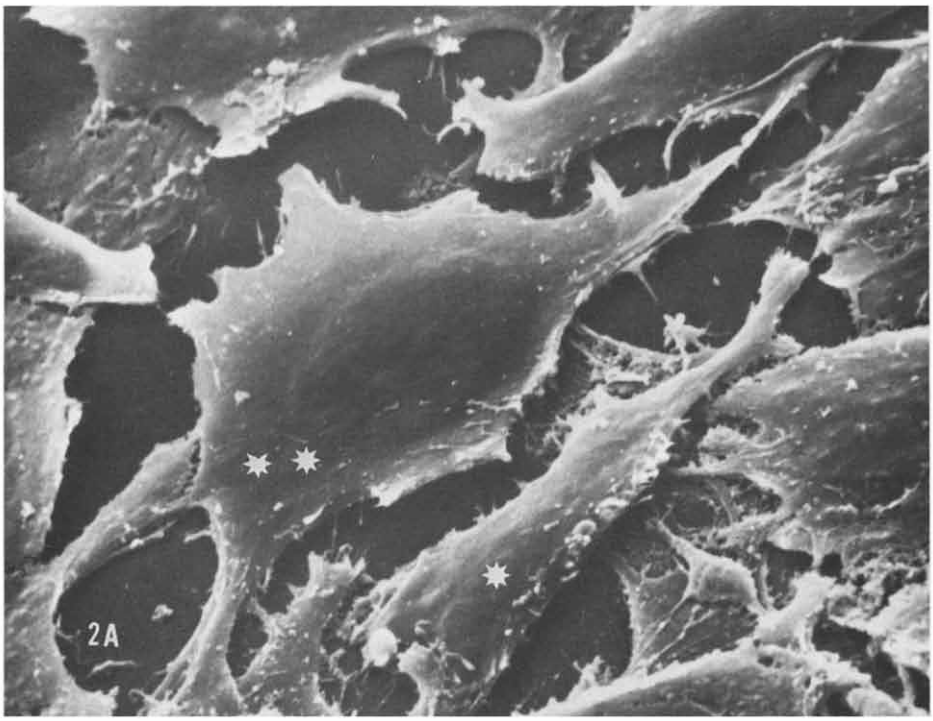
Using these two membrane isolation procedures we hoped to compare the composition of the whole plasma membrane (isolated via the technique of Brunette and Till) with the composition of the upper surface of the cell derived by the isolation technique of Barland and Schroeder. However, as will be seen below, the membrane isolated by the technique of Barland and Schroeder does not represent the entire upper surface of the cell.

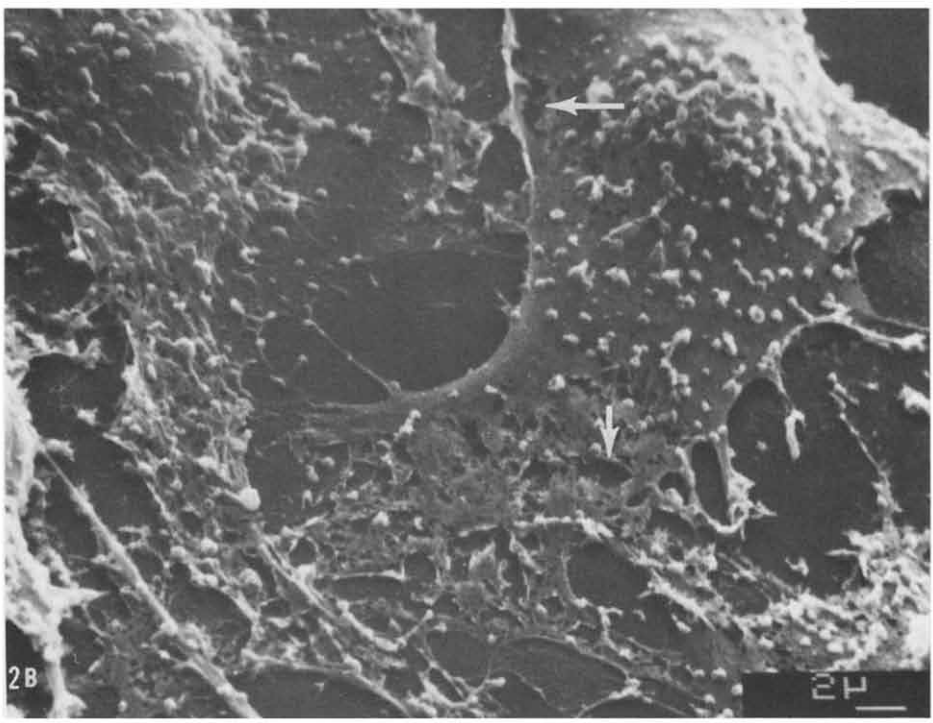
Morpholical examination of material isolated by the Barland and Schroeder membrane isolation technique. Barland and Schroeder [6] as well as Pitot and his associates [21], have published transmission electron micrographs demonstratint that at least a portion of the upper surface of a cell is removed by the membrane isolation technique originally described by Barland and Schroeder [6]. We have also used transmission electron microscopy to demonstrate that some cells in a cell population release a portion of their upper membrane following manipulation of the cell population according to the technique of Barland and Schroeder. We have extended these initial findings by applying scanning electron microscopy to the cell population in order to better identify that fraction of the upper surface of the cell which is removed when cells are subjected to this isolation procedure.

Fig. 2A is a scanning electron micrograph of sptr 3T3 cells grown to approx. 80% confluency, washed with phosphate-buffered saline and then fixed and prepared for microscopy. As can be seen, the cells in the sptr 3T3 cell culture display few blebs or microvilli. Differences in morphology can, however, be seen between individual cells. This heterogeneity in morphology is particularly evident between the cell in Fig. 2A marked with one star (fibroblastic) and the cell marked with two stars (epithelioid). This difference in morphology is apparently a critical factor in whether a cell will yield a membrane fraction when subjected to the procedures of Barland and Schroeder [6]. That this is the case is shown more clearly in Fig. 2G.

The addition of $ZnCl_2/Me_2SO$ to the sptr 3T3 cells (10 min, room temperature) results in a very striking increase in the number of blebs on the cell surface (Fig. 2B and C). These blebs have been seen on all cells examined, regardless of the morphology of the individual cell. Besides blebbing, many of the cells treated with $ZnCl_2/Me_2SO$ appear to contract; apparently stretching the membrane which lies around the cell edges (data not shown). Furthermore, some of the cells examined after $ZnCl_2/Me_2SO$ treatment display obvious holes in their upper surface (Fig. 2B, arrows).

Incubating the $ZnCl_2/Me_2SO$-treated cells in fluorescein mercuric acetate (40 min, 0°C) reduces the number of blebs on the cell surface, increases the number of holes in the upper membrane and, in some cells, apparently lifts the upper membrane away from the lower, substratum-attached membrane (Fig. 2D). Applying a shearing action to the fluorescein mercuric acetate-treated cells lifts a piece of membrane from the upper surface of the cells in the cell population. Those cells which do release part of their membrane seem to release that portion of the membrane which circumscribes the nucleus (Fig. 2E) leaving behind some upper membrane at the cell edges (Fig. 2E, arrow); the lower, substratum-apposed membrane (Fig. 2F, arrow) and a membranous 'cap' situated over the nucleus (Fig. 2F, star). That this 'cap' is indeed a membranous 'cap' can be better seen when isolated nuclei (not shown)





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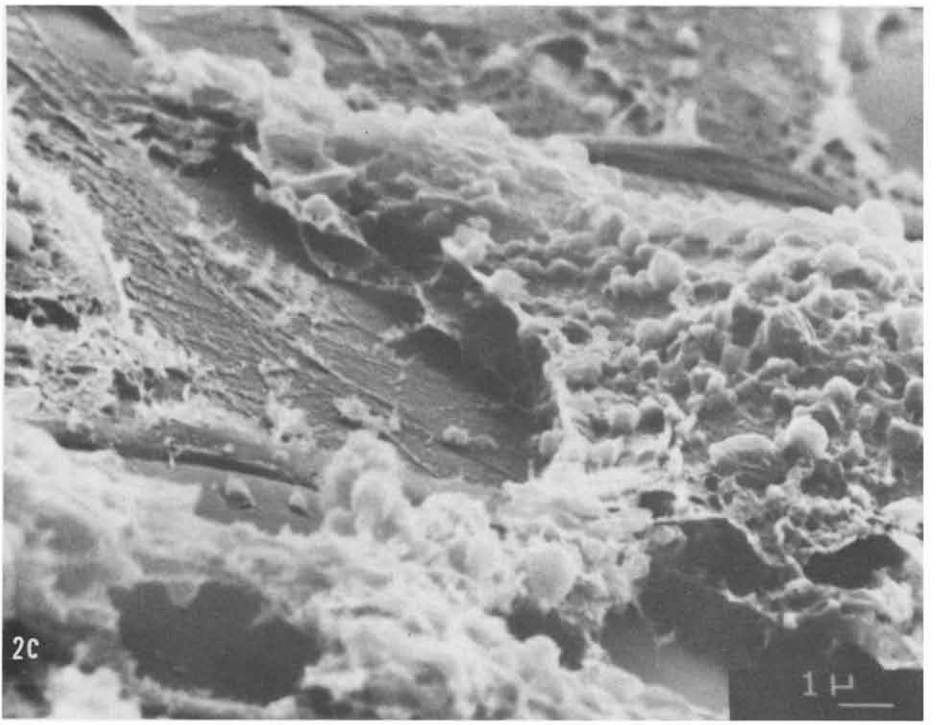

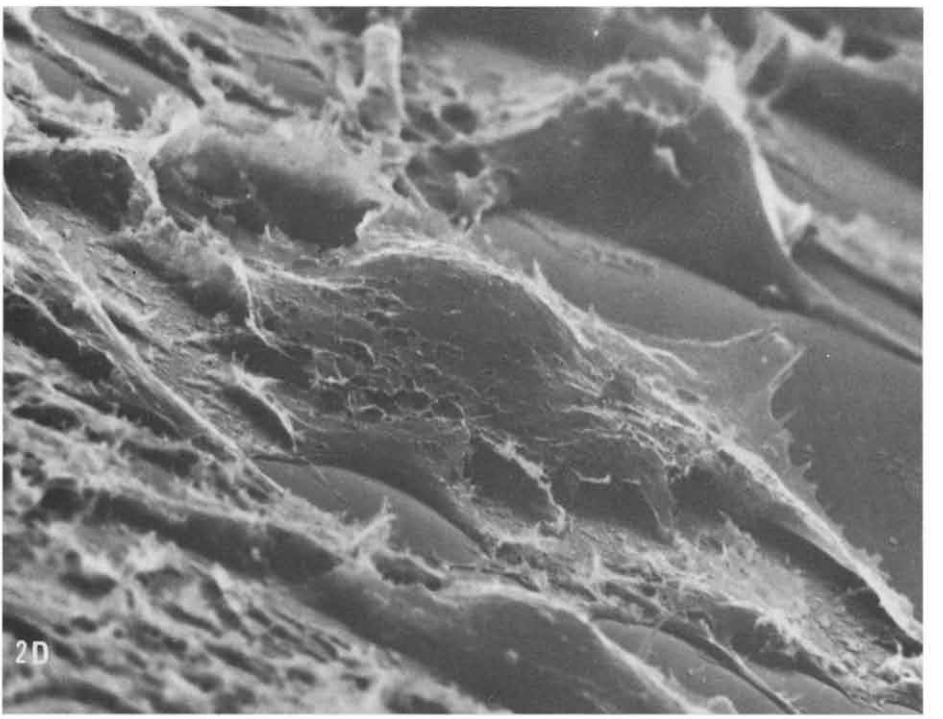

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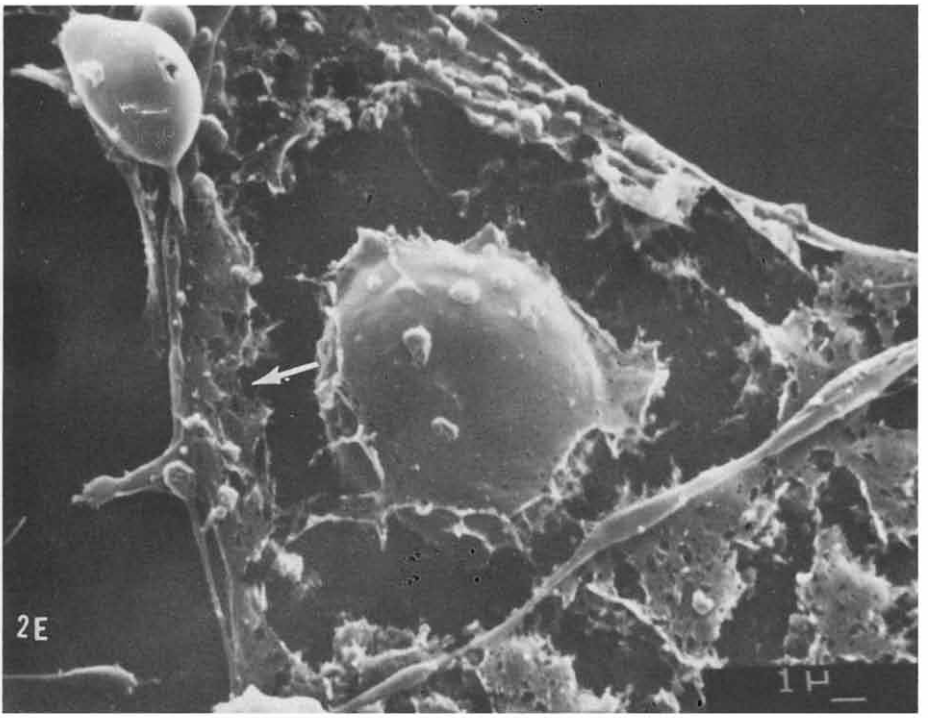
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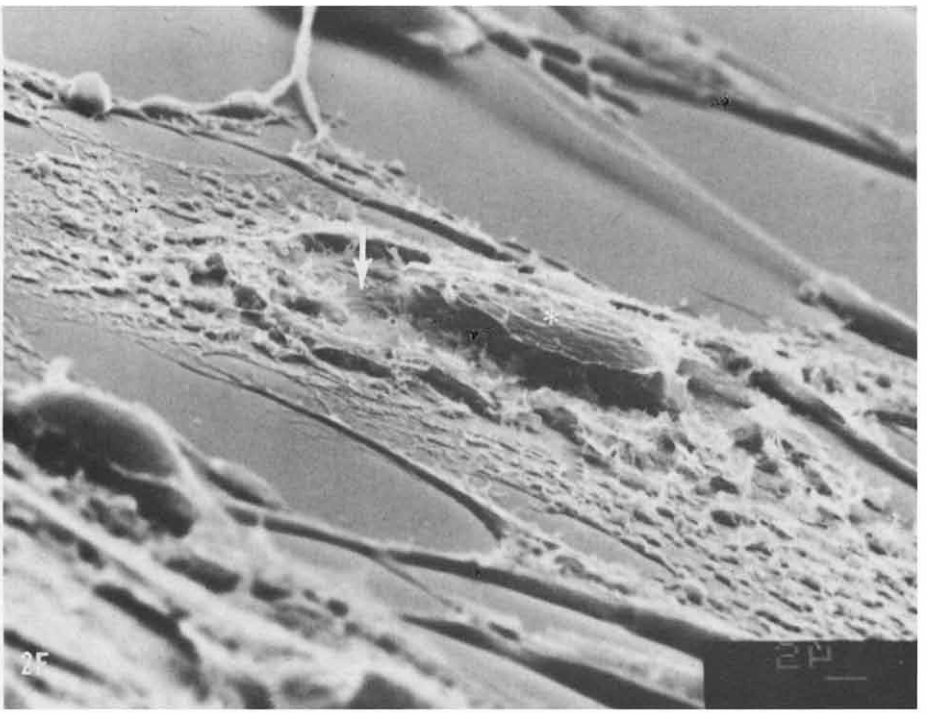
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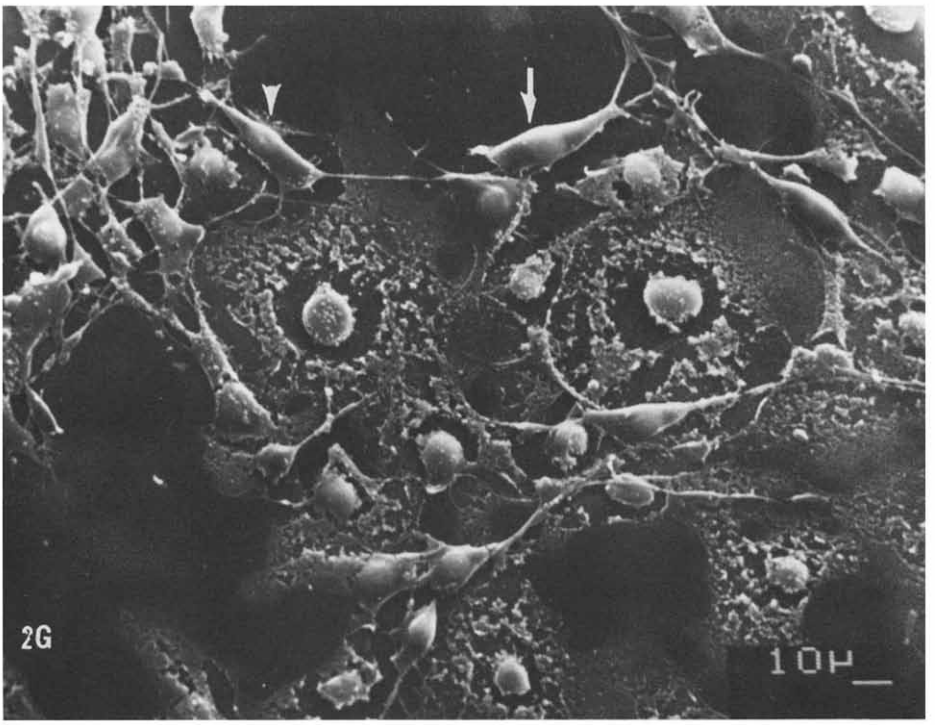


Fig. 2. A. Scanning electron micrograph of sptr 3T3 cells grown to 80% confluency, washed with phosphate-buffered saline and then fixed as described in Materials and Methods (X2000). B. sptr 3T3 cells washed with phosphate-buffered saline and then incubated 10 min at room temperature in $ZnCl_2/Me_2SO$ (X3000). C. As B, (X6000). D. sptr 3T3 cells washed with phosphate-buffered saline, incubated 10 min at room temperature in $ZnCl_2/Me_2SO$ and then allowed to stand in fluorescein mercuric acetate for 40 min at 0°C (X3000). E. sptr 3T3 cells washed with phosphate-buffered saline, incubated 10 min at room temperature in $ZnCl_2/Me_2SO$ and then shaken for 40 min in fluorescein mercuric acetate at 0°C (X4000). F. As E, (X2500). G. As E and F, (X500).

are compared to the nuclei which remain associated with the cells. The isolated nuclei are extremely smooth and show no evidence of the membrane which is situated over the nucleus in Figs. 2E and 2F.

It is extremely important to note that only those cells which display a flattened, epithelioid morphology following the initial phosphate-buffered saline wash release a membrane fraction (Fig. 2G). The more fibroblastic cells do not appear to release any membrane (Fig. 2G, arrows). If one increases the percentage of epithelioid cells within the cell population by decreasing the serum concentration in the growth medium then the number of cells releasing a membrane fraction increases proportionately to the percent epithelioid cells in the culture (data not shown).

Peptide composition of the isolated membranes. Fig. 3 is a Coomassie Blue stained SDS-polyacrylamide gel on which the peptides and glycopeptides of the homogenate, and the membrane fractions derived from the procedures of Barland and Schroeder [6] and Brunette and Till [7] have been separated. Although the stained profile of both membrane fractions clearly differ from that of the homogenate, there is an identity in many of the Coomassie Blue

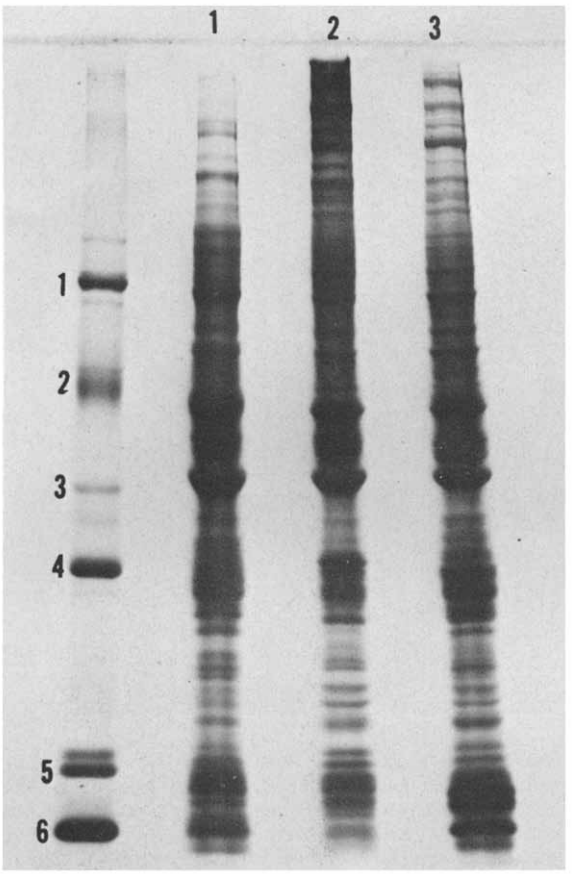
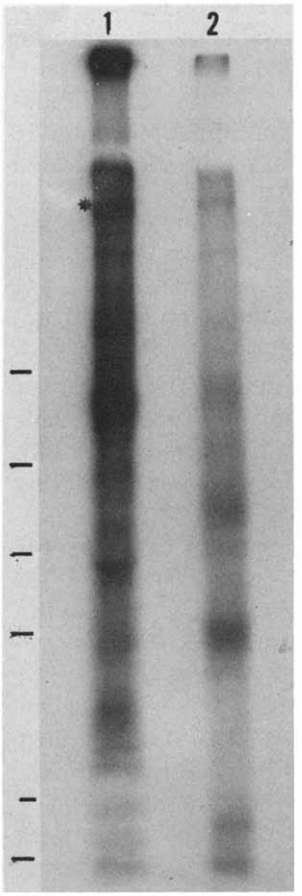


Fig. 3. Coomassie Blue staining of 7.5—12.5% linear SDS-polyacrylamide gel. From the left to right: molecular weight markers (1, phosphorylase *a*, 100 000; 2, bovine serum albumin, 67 000; 3, ovalbumin, 43 000; 4, DNAase I, 31 000; 5, soybean trypsin inhibitor, 21 500; 6, cytochrome *c*, 12 400). Lane 1, membrane fraction isolated according to Barland and Schroeder; lane 2, membrane fraction isolated according to Brunette and Till; lane 3, homogenate. 50 μg protein was applied to each lane. The homogenate was digested with 100 μg/ml DNAase I and RNAase (20 min, room temperature) prior to solubilization in SDS.

staining bands between the two membrane isolates. However, from the gel displayed in Fig. 3 it is evident that there are compositional differences between the two membrane fractions. It is particularly interesting to us that the higher molecular weight Coomassie Blue staining bands are much less prominent in the membrane fraction isolated according to Barland and Schroeder [6] as compared to the membrane fraction isolated according to Brunette and Till [7].

Iodinateable surface components. Fig. 4 is an autoradiograph of membrane peptides and glycopeptides which have been iodinated via the technique of Phillips and Morrison [14], isolated as described by Barland and Schroeder [6] or Brunette and Till [7] and then separated on a 7.5—12.5% SDS-polyacrylamide gel. As can be seen, the membrane fraction derived according to the technique of Brunette and Till contains a variety of iodinated components



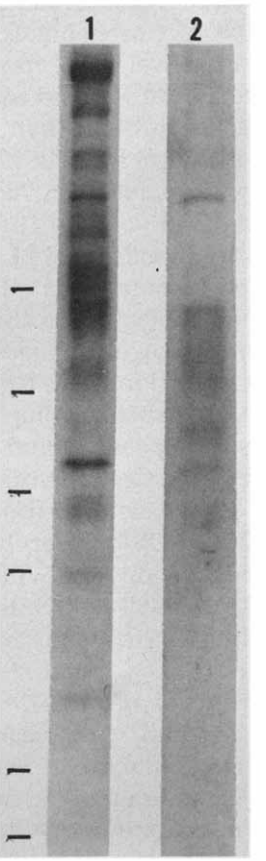


Fig. 4. Autoradiograph of ^{125}I-labeled membrane fractions. sptr 3T3 cells were labeled with ^{125}I while still atached to the substratum. Following iodination, membranes were isolated from separate cell populations according to the technique of Barland and Schroeder or Brunette and Till and then solubilized in Laemmli sample buffer. 20 000 cpm was applied to each well, the iodinated species separated on a 7.5—12.5% linear SDS-polyacrylamide gel and the iodinated species identified by autoradiography. From left to right: markers coinciding with molecular weight standards shown in Fig. 3; lane 1, membranes isolated as described by Brunette and Till; lane 2, membranes isolated as described by Barland and Schroeder; star, labeled band corresponding to fibronectin.

Fig. 5. Autoradiograph of SDS-polyacrylamide gel incubated with ^{125}I-labeled concanavalin A. Membrane fractions were isolated from sptr 3T3 cells grown to approximately 80% confluency. The isolated fractions were solubilized in Laemmli sample buffer and then 50 μg protein samples applied to a 7.5—12.5% SDS-polyacrylamide gel. Following staining the gel was overlayed with ^{125}I-labeled concanavalin A according to the modification of the technique described by Burridge [15]. The concanavalin A-binding glycopeptides were displayed by autoradiography of the dried gel. From left to right: molecular weight markers corresponding to the standards shown in Fig. 3; lane 1, membranes isolated as described by Brunette and Till; lane 2, membranes isolated as described by Barland and Schroeder.

which are either not found in the membrane fraction derived according to Barland and Schroeder or are present in such low amounts that they fail to show up well on the autoradiogram. It is worth noting that the membranes derived using the aqueous polymer technique [7] contain more fibronectin [22] (or large, external, transformation-sensitive protein [23], (star)) per mg

protein than does the membrane fraction derived after fluorescein mercuric acetate treatment of the cells. Evidence from a variety of other laboratories supports a localization of fibronectin to the cell edges [24] or the underside of the cell [25]. Quantitatively we have demonstrated that the membranes isolated via the procedure of Barland and Schroeder [6] contain approximately 3.2 times less ^{125}I per mg protein than does the membranous fraction isolated via the technique of Brunette and Till [7].

Glycopeptides of the membrane fractions. Quantitatively we have demonstrated that the specific activity of [^{3}H]glucosamine-labeled membranes isolated via the Brunette and Till [7] technique is 20 times higher than the specific activity of membranes isolated from cells maintained under the same growth conditions but subjected to the Barland and Schroeder [6] isolation procedure. The same relative differences in specific activity have been obtained from cells labeled with [^{3}H]fucose. It must be noted that both glycoproteins and glycolipids would be labeled using these two carbohydrate precursors and, therefore, both molecular species would be taken into account when comparing the specific activities of the two membrane isolates.

The binding of ^{125}I-labeled concanavalin A to membrane components separated on 7.5—12.5% SDS-polyacrylamide gels (Fig. 5) displays approx. 20 concanavalin A-binding glycopeptides in the membrane fraction isolated via the procedure of Brunette and Till [7] while detecting only approx. 6 concanavalin A-binding glycopeptides in the membrane fraction isolated via the procedure of Barland and Schroeder [6].

Relative lipid composition of the two membrane fractions. One of our earliest and most surprising findings with regard to the properties of the membrane fraction isolated via the Barland and Schroeder technique was the high density displayed by these membranes in sucrose. Specifically when membranes isolated according to the technique of Barland and Schroeder were resuspended in 55% (w/w) sucrose, overlayered with a linear sucrose gradient ranging from 22 to 50% (w/w) sucrose and centrifuged at approx. 110 000 $\times g$ for 18 h, the membranes remained in the 55% sucrose cushion (δ = 1.2619). Membranes isolated via the technique of Brunette and Till, when resuspended in 55% (w/w) sucrose, overlayered with a 23—53% (w/w) linear sucrose gradient and centrifuged 18 h at 110 000 $\times g$, spun up into a tight band at approx. 36% (w/w) sucrose (δ = 1.1566). Furthermore, membranes isolated via the Brunette and Till technique and then treated with $ZnCl_2$/Me_2SO-fluorescein mercuric acetate, as described in the Barland and Schroeder membrane isolation technique, floated up in an identical gradient into a band at approx. 45% (w/w) sucrose (δ = 1.2090).

In order to determine whether the apparent density of the membranes isolated according to the technique of Barland and Schroeder might be related to an altered protein to lipid ratio, the fluorescein mercuric actate-treated membranes were extracted with chloroform/methanol, phospholipid phosphorus determined and compared to the values obtained from chloroform/methanol extracts of membranes isolated via the Brunette and Till technique. Such an analysis yielded a value of 15.6 μg phospholipid phosphorus/mg protein for the membranes isolated according to Barland and Schroeder and 275 μg phospholipid phosphorus/mg protein for membranes isolated via the

two phase polymer technique. These data suggest a 17-fold enrichment in phospholipid phosphorus in the membrane fraction derived via the Brunette and Till technique relative to the fraction obtained from the Barland and Schroeder technique. Preliminary evidence using radiolabeled phospholipid precursors has supported this extreme asymmetry in the protein to lipid ratios in the two membranes isolated (data not shown).

Extraction of membrane components by $ZnCl_2$/Me_2SO-fluorescein mercuric acetate. One possible explanation of the compositional differences observed between the membranes isolated via the Barland and Schroeder technique [6] and those isolated via the Brunette and Till technique [7] would be that the $ZnCl_2$/Me_2SO-fluorescein mercuric acetate pre-treatment of the cells required by the Barland and Schroeder method extracts components of the cell membrane. In our early work directed at addressing this potential problem, we demonstrated that the Coomassie Blue staining profile of a Brunette and Till [7] membrane fraction isolated from sptr 3T3 cells treated with $ZnCl_2$/Me_2SO-fluorescein mercuric acetate, as in the Barland and Schroeder [6] technique, was the same as the Coomassie Blue staining profile obtained from sptr 3T3 cells isolated without pre-treatment (data not shown). These data suggested that no preferential extraction of specific membrane peptides resulted from the pre-treatments. These data could not, however, rule out a quantitative, as opposed to a qualitative, extraction of membrane proteins or glycoproteins. To rule out quantitative extraction of membrane components via the $ZnCl_2$/Me_2SO-fluorescein mercuric acetate pre-treatment, cells were radiolabeled with [^{3}H]leucine, [^{3}H]glucosamine or [^{3}H]choline, as described in Materials and Methods, and the relative extraction of membrane peptides, glycopeptides or phospholipids determined. This question has become particularly important with regard to the relative extraction of phospholipids since Pitot and his collaborators [21] have suggested that the high protein to lipid ratio found in the membranes isolated via the technique of Barland and Schroeder results from the extraction of phospholipids.

Table II demonstrates that the non-dailyzable leucine counts extracted into the $ZnCl_2$/Me_2SO wash amounts to only 0.1% of the total cell counts, whereas 21% of the total leucine counts are extracted into fluorescein mercuric acetate (40 min, 0°C). However, a fluorograph (not shown) of the fluorescein mercuric acetate-extracted, non-dialyzable leucine-labeled material separated on a 7.5—12.5% SDS-polyacrylamide gel demonstrated that the material extracted into the fluorescein mercuric acetate did not represent a preferential extraction of material corresponding to the Coomassie Blue staining bands missing from the membranes isolated according to Barland and Schroeder [6]. $ZnCl_2$/Me_2SO extraction of [^{3}H]glucosamine-labeled cells removes 0.2% of the total cell label into nondialyzable material, while fluorescein mercuric acetate treatment extracts 7% of the total cell counts (Table II). Again fluorograhy of the extracted [^{3}H]glucosamine-labeled material failed to indicate a preferential extraction of material which co-migrates with the components missing from the membranes isolated according to Barland and Schroeder (data not shown). $ZnCl_2$/Me_2SO extraction of [^{3}H]choline-labeled whole cells removed 14% of the total cell counts while fluorescein mercuric acetate treatment extracted another 16% of the [^{3}H]choline counts. Although it is not feasible to dialyze

TABLE II

EXTRACTION OF LABELED MATERIAL FROM WHOLE CELLS

Cells were grown for 72 h in the presence of the indicated precursors. The cells were then washed 5 times with phosphate-buffered saline. The number of counts released into the fifth wash was determined. Following the phosphate-buffered saline wash the same cells were incubated for 10 min at 22°C with $ZnCl_2$/Me_2SO and the counts released determined. Finally the same cells were incubated 40 min at 0°C (without shaking) in fluorescein mercuric acetate and the counts released determined. The released leucine- and glucosamine-labeled material was dialyzed in an effort to remove any free radioisotope which was released from soluble pools. The material released from choline-labeled cells was not dialyzed for fear of losing small lipid micelles along with any free choline pool. Care was taken to account for any quenching of the samples by $ZnCl_2$/Me_2SO and fluorescein mercuric acetate. The data presented represent cpm corrected for quench. It should be noted that we have not demonstrated that leucine labels only peptides and glycopeptides, that glucosamine labels only glycopeptides and glycolipids or that choline labels only phospholipids. Therefore, these data should be considered only as percent total label extracted and should not be taken as exact determinations of the percent protein, glycoprotein or phospholipid extracted. n.d., not determined.

Radioactive precursor	Treatment					
	Phosphate-buffered saline (% of whole cells)		$ZnCl_2$/Me_2SO (% of whole cells)		Fluorescein mercuric acetate (% of whole cells)	
	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis
[^{3}H]Leucine	0.4	0.1	2	0.1	29	21
[^{3}H]Glucosamine	0.5	0.1	6	0.2	29	7
[^{3}H]Choline	0.9	n.d.	14	n.d.	16	n.d.

[^{3}H]choline-labeled material, it is virtually certain that some of the extracted label represents material from the soluble pool.

The data generated from these protocols could be taken to indicate actual extraction of membrane components. Alternatively the 'extracted' material could represent the leakage of soluble intracellular pools and cytoplasmic components through the holes in the membrane produced by $ZnCl_2$/Me_2SO and fluorescein mercuric acetate treatments (see Fig. 2, A—G). In order to distinguish these two possibilities, radiolabeled membranes were isolated via the Brunette and Till technique and then treated sequentially with $ZnCl_2$/Me_2SO and fluorescein mercuric acetate. Table II contains the accumulated data. Treatment of [^{3}H]leucine-labeled membranes with phosphate-buffered saline (phosphate-buffered saline$_1$; 10 min, room temperature) removed 1.7% of the total radiolabel associated with the membrane while treatment with $ZnCl_2$/Me_2SO (10 min, room temperature) removed 2.5% of the label. Further treatment of the same membranes with phosphate-buffered saline (phosphate-buffered saline$_2$; 40 min, 0°C) extracted 3.1% of the total counts while fluorescein mercuric acetate treatment (40 min, 0°C) removed 3.9% of the counts. Treatment of [^{3}H]glucosamine-labeled membranes with phosphate-buffered saline (phosphate-buffered saline$_1$; 10 min, room temperature) removed 6.1% of the total counts, while treatment with $ZnCl_2$/Me_2SO (10 min, room temperature) removed only 2.3% of the counts. A second incubation in phosphate-buffered saline (phosphate-buffered saline$_2$; 40 min, 0°C) removed another 2.3% of the total glucosamine label, while fluorescein mercuric acetate treatment of the $ZnCl_2$/Me_2SO-treated membranes removed 3.8% of the counts.

TABLE III

EXTRACTION OF LABELED MATERIAL FROM MEMBRANES ISOLATED ACCORDING TO BRUNETTE AND TILL

Cells were grown for 72 h in the presence of the labeled precursor. The membrane fraction was then isolated according to the technique of Brunette and Till [7]. The membranes were all washed once with phosphate-buffered saline and then incubated 10 min at 22°C in phosphate-buffered saline$_1$ or $ZnCl_2$/Me_2SO. The membranes were pelleted and the percent extracted material determined. The pellet was resuspended in phosphate-buffered saline$_2$ or fluorescein mercuric acetated and incubated 40 min at 0°C. The membranes were again pelleted and the percent material extracted determined. Care was taken to account for any quenching of the samples by $ZnCl/Me_2SO$ and fluorescein mercuric acetate. The data presented represent cpm corrected for quench. That the leucine and glucosamine have labeled membrane peptides and glycopeptides, respectively has been demonstrated by the fact that more than 95% of the leucine label and 90% of the glucosamine label can be precipitated by 1 h incubation of the isolated membranes in ice-cold 10% trichloroacetic acid. That the choline has preferentially labeled membrane phospholipids has been demonstrated by the fact that more than 90% of the counts present in the isolated membrane fraction can be extracted into the non-aqueous phase of chloroform/methanol.

Treatment	Radioactive precursor		
	[^{3}H]Leucine (% of untreated membrane)	[^{3}H] Glucosamine (% of untreated membrane)	[^{3}H] Choline (% of untreated membrane)
Phosphate-buffered saline$_1$	1.7	6.1	1.1
$ZnCl_2$	2.5	2.3	2.4
Phosphate-buffered saline$_2$	3.1	2.3	0.6
Fluorescein mercuric acetate	3.9	3.8	2.2

Treatment of [^{3}H]choline-labeled membranes with phosphate-buffered saline (phosphate-buffered saline$_1$; 10 min, room temperature) removed 1.1% of the total membrane associated counts, while $ZnCl_2/Me_2SO$ (10 min, room temperature) removed 2.4% of the counts. A second phosphate-buffered saline wash (phosphate-buffered saline$_2$; 40 min, 0°C) removed another 0.6% of the tritium, while fluorescein mercuric acetate treatment (40 min, 0°C) removed 2.2% of the total label. Thus, in each instance $ZnCl_2/Me_2SO$ plus fluorescein mercuric acetate treatment removed 5—6% of the total label associated with the membrane while sequential incubations in phosphate-buffered saline removed between 3 and 7% of the labeled material. These data suggest to us that neither the $ZnCl_2/Me_2SO$ not the fluorescein mercuric acetate pre-treatment of the membranes extracts significant quantities of membranous material.

It could be argued that membrane components (in particular, lipid-rich vesicles) are extracted only when the cells are attached to the substratum and are able to bleb (see Fig. 2C and D). Evidence that lipid-rich vesicles are not extracted from cells associated with the substratum is presented in Fig. 6, A—C. sptr 3T3 cells were grown for 72 h in 2.5 μCi/ml ortho[^{32}P]phosphate to label the phospholipids. Cells were then washed with 0.16 M NaCl + 0.01% $CaCl_2$, followed by incubation in $ZnCl_2/Me_2SO$ (10 min, room temperature) and then fluorescein mercuric acetate (40 min, 0°C, no shaking). The supernatant from each treatment was brought to 55% (w/w) sucrose and overlayered with a 10—35% (w/w) linear sucrose gradient. The gradient was centrifuged 4 h at 48 000 $\times g$ and then fractionated. It would be expected that lipid-rich vesicles

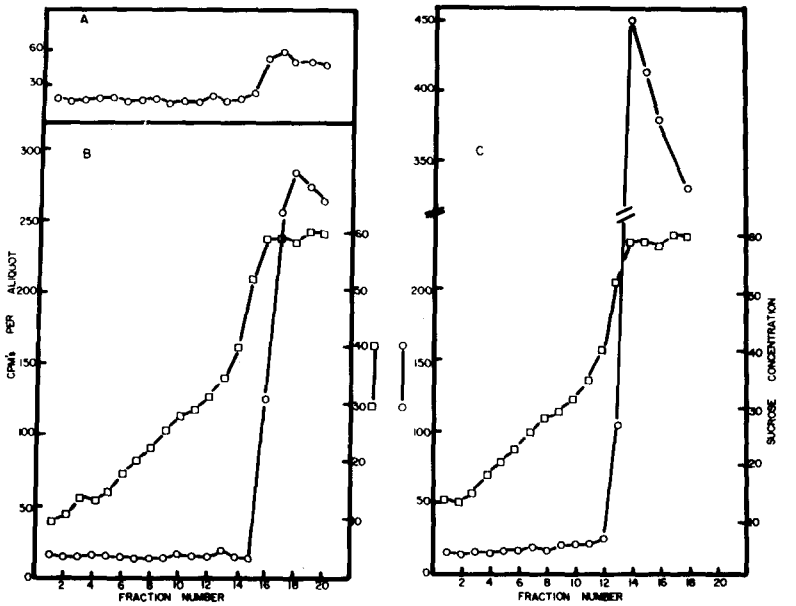


Fig. 6. Profile of three sucrose gradients intended to display any lipid-rich vesicles released from the cells treated as described. A. Cells were washed with 0.16 M NaCl + 0.01% $CaCl_2$. The supernate was then brought to 55% (w/w) sucrose and overlayered with a 10—35% (w/w) linear sucrose gradient. Points indicate cpm ^{32}P. B. Cells were incubated in $ZnCl_2/Me_2SO$ (10 min, room temperature). The supernatant was then brought to 55% (w/w) sucrose and overlayed with a 10—35% (w/w) linear sucrose gradient. ○———○, cpm ^{32}P; □———□, sucrose concentration (determined from refractive index). C. Cells were incubated in fluorescein mercuric acetate (40 min, 0° C). The supernatant was then brought to 55% (w/w) sucrose and overlayed with 10—35% (w/w) linear sucrose gradient. ○———○, cpm ^{32}P; □———□, sucrose concentration (determined from refractive index). Each fraction off the gradient was 2.0 ml. The cpm ^{32}P per 10 μl are displayed on the graph. The sucrose density gradient was the same in both A and B.

released during any of the three washings would move out of the high density sucrose cushion and be found in the linear portion of the gradient. As can be seen (Fig. 6, A—C) there is no evidence of the movement of any ^{32}P-labeled components into the lower density regions of the sucrose gradient, suggesting that few, if any, lipid-rich vesicles are extracted during the various washing procedures employed in the isolation of membranes via the Barland and Schroeder [6] technique.

Demonstration that membrane components are metabolic products of the cell. In order to demonstrate that the components isolated in the membrane fraction derived by the Barland and Schroeder technique were synthesized by the cells, cells were grown for 72 h in 2.5 μCi/ml [^{3}H]leucine, the membranes isolated, and membrane peptides and glycopeptides separated on a 7.5—12.5% SDS-polyacrylamide gel. A fluorograph (not shown) clearly demonstrated that all the major Coomassie Blue staining bands were labeled, suggesting that the components of the isolate are metabolic products of the cell and not simply a $ZnCl_2/Me_2SO$-fluorescein mercuric acetate-induced aggregation of components from the medium. Similarly, growing the cells for 72 h in 4.0 μCi/ml [^{3}H]proline and then isolating the membranes via the technique of Barland and Schroeder [6] prior to separation of the components on an SDS-polyacrylamide gel, produced a fluorograph (not shown) in which all the major

Coomassie Blue-stained bands were labeled. Significantly, [^{3}H]proline did not preferentially label components migrating at the same apparent molecular weights as rat tail collagen standards which were co-electrophoresed with the proline-lalbeled isolate, suggesting that the membrane does not represent an aggregate of collagen and cell protein.

In a similar experiment sptr 3T3 cells were grown for 72 h in ^{125}I-labeled calf serum and then the membrane-enriched fraction isolated according to the technique of Barland and Schroeder. From our data (not shown) it is clear that much less than 1 μg iodinated serum protein could be associated with 1 mg membrane protein and, therefore, none of the major Coomassie Blue stained bands in Fig. 3 could represent contamination of the membrane isolate with serum components.

Discussion

In this manuscript we have addressed ourselves to identifying and characterizing that part of the plasma which is removed from substratum-associated cells during the membrane isolation technique originally described by Barland and Schroeder [6]. The data derived from our scanning electron micrographs demonstrate that this isolation technique releases an area of the upper cell surface from a percentage of morphologically distinct cells in the cell population. The part of the upper cell surface which is released circumscribes the nucleus. A 'cap' of upper membrane surface, as well as parts of the upper surface extending onto the cell edges, remain associated with those cells from which the membrane has been removed. Those cells which yield a membrane fraction also leave the substratum-apposed membrane as well as the nucleus associated with the surface of the tissue culture dish.

It must be realized that within the population of the cells described in this manuscript only approximately 50% of the cells yield a membrane fraction when subjected to the isolation procedure of Barland and Schroeder. Those cells which yield this membrane fraction are the more flattened, epithelioid cells in the population. It is not clear from the data we have gathered to date whether this epithelioid morphology provides a more amenable substrate for the $ZnCl_2$/Me_2SO-fluorescein mercuric acetate solution to act on or whether the stretching which is more pronounced in the flattened cells imparts a mechanical inflexibility to the membrane thereby increasing the likelihood that a part of the upper surface of the cell will be sheared free of the remaining membrane during agitation. It is also unclear whether the morphologic diversity seen among the various cells in the sptr 3T3 cell culture represents morphologic variants within the cell population or the same cells at different stages of the cell cycle [26]. This point is presently being studied in our laboratory.

It is of considerable interest to us that our data suggest that compositional differences exist between membranes isolated according to Barland and Schroeder and Brunette and Till. These compositional differences are particularly obvious among the high molecular weights peptides and glycopeptides separated on SDS-polyacrylamide gels. We believe that the data presented in this manuscript strongly suggest that the differences in membrane peptide and glycopeptide composition observed between the two membrane isolates do not

result from extraction of membranous components via the $ZnCl_2/Me_2SO$-fluorescein mercuric acetate treatment. However, it is our opinion that the compositional differences observed between the two membrane fractions could result from a compositional differentiation of topologically distinct areas of the plasma membrane, be an inherent feature of cells with an epithelioid morphology and, therefore, not generalizable to all cell shapes or even be the result of a $ZnCl_2/Me_2SO$-fluorescein mercuric acetate-induced redistribution of membrane components. The latter hypothesis would suggest that the compositional differences observed between the two membrane fractions are artifacts of the $ZnCl_2/Me_2SO$-fluorescein mercuric acetate treatment. This possibility must be very seriously considered with regard to the membrane phospholipids since our data would suggest that the membrane isolated via the technique of Barland and Schroeder would have the highest protein to lipid ratio of any membrane reported to date. The possibility that membrane peptides or glycopeptides are translocated in the plane of the membrane as a result of the $ZnCl_2/Me_2SO$-fluorescein mercuric acetate treatment can be tested with probes directed at membrane components which our data suggest are restricted to the membrane fraction released during the Barland and Schroeder isolation procedure. We have chosen to use antisera against a number of the cancanavalin A receptors which we have identified as being localized to this membrane 'compartment' to test this hypothesis.

Among the points made in this paper we believe one stands out most clearly. Specifically the plasma membrane isolation technique originally described by Barland and Schroeder [6] does not isolate a membranous fraction which is representative of the overall structure of the plasma membrane. It is our opinion that, as a result of its selectivity with regard to cell morphology and area of the plasma membrane isolated, this isolation procedure probably has only limited application to the study of generalized membrane structure. Furthermore, in light of our work we would suggest that some of the data which has been collected by other laboratories [27,28] using this isolation technique should be re-evaluated. However, we do believe that this isolation technique might, after further study, provide a tool for investigating the mechanism(s) whereby a cell retains specific membrane components in distinct compartments vis á vis the solid substratum to which the cells adhere.

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