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Isolation of Plasma Membranes from Human Skin Fibroblasts

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Summary. Plasma membranes have been isolated on a large scale from cultured human skin fibroblasts by methods which avoid treatment with either proteases or heavy metals. Cells ($0.5–1.0 \times 10^9$) were harvested from roller bottles and disrupted by manual agitation in hypotonic medium. A plasma membrane-enriched fraction (10–15 mg protein) was then obtained by conventional differential and density gradient centrifugation. Fractionation was monitored by phase-contrast and electron microscopy, measurement of activities of marker enzymes and recovery of ^{125}I wheat germ agglutinin which was bound to the cell surface prior to disruption. The membranes obtained were mainly vesicular (0.1–2 μm diameter) and banded at a sucrose density of 1.12. 5′-Nucleotidase was purified approximately 20-fold with respect to the cell homogenate and nearly 50% of this enzyme was recovered in the plasma membrane-enriched fraction. Activities of Mg^{2+}-ATPase, $(Na^+ + K^+)Mg^{2+}$-ATPase and prostaglandin E, and fluoride-stimulated adenylate cyclases were also maximal in this fraction. Recovery and specific activity of ^{125}I wheat germ agglutinin bound to the cell surface was greatest in these membranes. The only contaminating structures identifiable in electron micrographs resembled the flat cisternal plates of the Golgi apparatus. The detection of sialyl and galactosyl transferase activities was consistent with the presence of these structures. Some further subfractionation of the various markers in the preparation was possible. A similar plasma membrane-enriched fraction also was obtained by a second method in which cells were harvested by scraping from the roller bottles and disrupted by homogenization in isotonic media.

There are several genetically determined human diseases which involve alterations in the surface plasma membrane of a variety of tissue cells. For example, in hypercholesterolemia (Brown & Goldstein, 1976), there is an absence of the cell surface receptor for low density lipoproteins and as a result the normal feedback inhibition of cholesterol biosynthesis is defective (Goldstein & Brown, 1976). Changes in erythrocyte membrane proteins have been demonstrated in hereditary spherocytosis (Engelhardt, 1976) and in muscular dystrophy (Roses, Roses, Miller, Hull & Appel, 1976). The significance of the latter observation is not yet understood. In a number of other diseases both genetic and acquired, changes in

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some aspects of membrane structure or function have been at least implicated (for partial survey, *see* Bolis, Hoffman & Leaf, 1976). Although considerable information about the properties of the plasma membranes can be gained from studies of the intact cell, detailed biochemical studies clearly require purification of the membranes.

Several methods for the isolation of plasma membranes from cultured cells are currently in use (Warren, Glick & Nass, 1966; Bosmann, Hagopian & Eylar, 1968; Barland & Schroeder, 1970; Perdue & Sneider, 1970; Brunette & Till, 1971; Bingham & Burke, 1972; Atkinson, 1973; Schimmel, Kent, Bischoft & Vagelos, 1973). Most widely used are methods based on the original procedure of Warren *et al.* (1966) which employs fixation of the cell surface with heavy metal prior to disruption. This treatment yields large fragments of the fixed cell surface membrane which can then be separated from other subcellular components on the basis of size and shape or surface characteristics. Effective use of the latter properties for plasma membrane isolation has been made by Brunette and Till (1971) in their aqueous two-phase polymer system. This method which has become widely used for various cell types (Lesko, Donlon, Marinetti & Hare, 1973; Sullivan & Jerry, 1974; Juliano & Gagalang, 1975) is dependent on fixation with a heavy metal, usually Zn^{2+}, so that large ghostlike fragments are obtained.

Our aim was to isolate plasma membranes on a sufficiently large scale to permit biochemical studies of their molecular constituents without the use of proteases for harvesting of cells or the use of fixatives which influence function. Two procedures employing conventional separation techniques were developed which satisfied these criteria. A preliminary report of this work has already appeared (Kartner, Alon, Swift, Buchwald & Riordan, 1976).

Materials and Methods

Materials

Roller bottles in which fibroblasts were grown were obtained from Bellco as were the large scrapers for harvesting cells. Cells were grown in α medium (Stanners, Eliceiri & Green, 1971) containing 15% fetal calf serum (Flow Laboratories). Phosphate buffered saline (PBS) consisted of 0.15 M NaCl and 0.01 M NaH_2PO_4, pH 7.4. AMP, ATP, GTP, glucose-6-phosphate and 2-(*p*-iodopenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium were from Sigma. Pyruvate kinase and phosphoenol pyruvate were from the same source. [8-^{14}C] AMP and [8-^{14}C] cyclic 3′, 5′ AMP were from Amersham/Searle and [α-^{32}P] ATP and $Na^{125}I$ came from New England Nuclear. Fluorescamine (4-phenylspiro[furan-2(3H), 1′-phthalan]3-3′dione) was purchased from Roche Diagnostics.

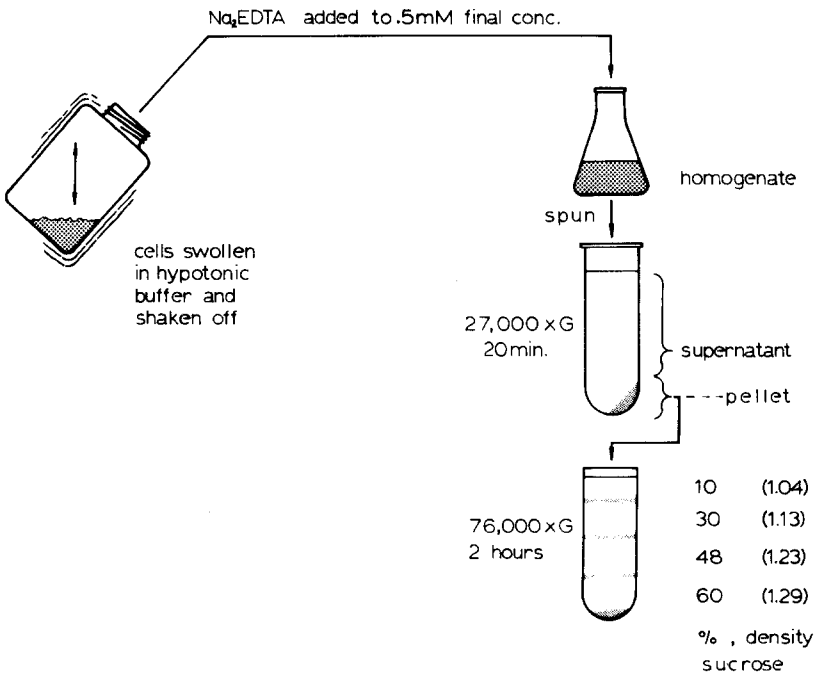

Fig. 1. Schematic representation of steps employed in first method of isolation of a plasma membrane-enriched fraction from cultured human fibroblasts

Methods

Cell Culture

The cell strains used in these experiments were derived from skin biopsies according to the method of Goldstein and Littlefield (1969). The cells were subcultured by trypsinization with 0.25% Bacto trypsin in citrate saline (134 mM tri-sodium citrate and 15 mM KCl, pH 7.8). Cells were stored frozen in 10% glycerol in α medium with fetal calf serum. The cells used for the isolation of membranes were grown from frozen stocks and subcultured 1:10 into roller bottles, grown for 10–14 days with twice weekly feedings.

Isolation of Membranes

Method One-Hypotonic Treatment. The procedure is outlined schematically in Fig. 1. Cells in each roller bottle were washed six times with 25 ml PBS. Washed cells were swollen by slowing rotating bottles containing 25 ml of 1 mM $NaHCO_3$, pH 7.4, for 1 min. This hypotonic medium was removed and the treatment repeated twice. The swollen cells could then be easily removed from the surface and disrupted by manually shaking the bottle containing 25 ml of 1 mM $NaHCO_3$. The disrupted suspension obtained in this way was made 0.5 mM in EDTA (ethylene diamine tetracetate, disodium salt) and centrifuged at $27{,}000 \times g$ for 20 min. The addition of EDTA is necessary to prevent aggregation and clumping of the various types of particulate material present, especially the sticking of

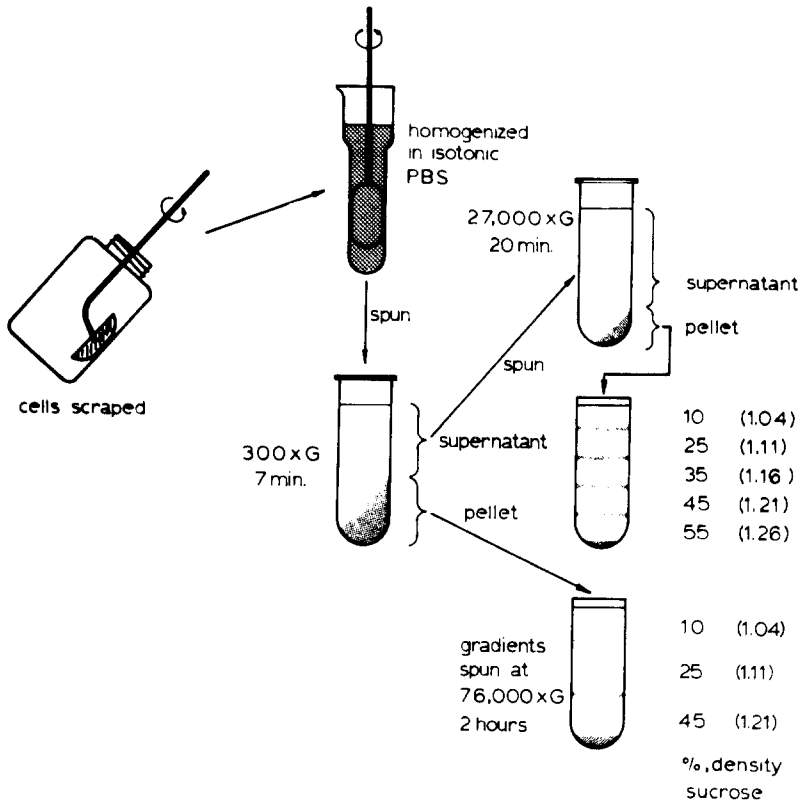

Fig. 2. Schematic representation of Method Two

membrane fragments to nuclei. However, if the EDTA is present during disruption, nuclear damage is extensive and considerable amounts of released chromatin are detected at subsequent stages of the isolation. The pellet obtained was resuspended in 10 ml of 10% (w/v) sucrose and layered on a discontinuous density gradient composed of 15 ml each of 30, 48 and 60% sucrose which was centrifuged at $76{,}000 \times g$ for 2 hr in an SW 25.2 rotor. Material banding at the interfaces between the sucrose layers was removed by aspiration, washed free of sucrose using 1 mM $NaHCO_3$ and pelleted by centrifugation at $33{,}000 \times g$ for 20 min. Pellets were resuspended in 1 mM $NaHCO_3$, aliquots taken for assays of proteins, enzymes and surface labels. The remainder was stored at -20 °C. Prior to the final resuspension, samples of pellets were taken for thin sections to be processed for electron microscopy.

Method Two-Isotonic Treatment. Fig. 2 schematically outlines this method. In order to harvest and disrupt cells under isotonic conditions, cells were scraped from the surface using a large scraper with a rubber blade and pelleted by centrifugation at $300 \times g$ for 7 min. After resuspension in 4 volumes of PBS, cells were homogenized using a ground glass homogenizer. The degree of homogenization was monitored by phase-contrast microscopy and terminated before disruption of nuclei occurred.

The isotonic homogenate was centrifuged at $300 \times g$ for 7 min. The supernatant was then spun at $27{,}000 \times g$ for 20 min to yield a pellet which, after resuspension in 10 ml of 10% sucrose, was layered on a sucrose density gradient consisting of 12 ml each of

25, 45 and 55% sucrose. The nuclear pellet from the low speed spin ($300 \times g$, 7 min) was also resuspended in 10% sucrose and layered on a two-step gradient of 25 and 45% sucrose. All tubes containing sucrose gradients were centrifuged at $76{,}000 \times g$ for 2 hr. Materials banding at the interfaces were treated as in Method One.

Electron microscopy

Portions of freshly prepared fractions were fixed in cold (2 °C) 2.7% glutaraldehyde in 0.07 M NaH_2PO_4, pH 7.4. After rinsing in the same buffer the fractions were postfixed with 1% uranyl acetate in 25% ethanol, dehydrated in graded ethanol solutions and embedded in spurr epoxy resin. Ultrathin sections were cut on a Porter Blum MT-2 ultramicrotome and viewed in a Phillips Em 201 electron microscope at 60 kV.

Enzyme Assays

5′-Nucleotidase activity was assayed at 37 °C in a solution containing 0.05 M Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 5 mM AMP. Tracer amounts of [8-^{14}]AMP permitted the conversion of AMP to adenosine to be followed radiochemically according to Suran (1973). Assays of Mg^{2+}-ATPase activities were performed in 0.05 M Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 5 mM ATP. $(Na^+ + K^+)Mg^{2+}$-ATPase activities were assayed in a solution of 0.1 M Tris-HCl, pH 7.5, 2 mM $MgSO_4$, 0.1 mM EGTA, 60 mM NaCl and 2 mM ATP in the presence or absence of 5 mM KCl. The specific activity of $(Na^+ + K^+)Mg^{2+}$-ATPase was obtained by subtraction of that in the latter solution from that in the former. Glucose-6-phosphatase activity was measured in 0.1 M maleic acid, pH 6.5, with 10 mM G-6-P as substrate. In ATPase and glucose-6-phosphatase assays, the amounts of inorganic phosphate liberated were measured (Allen, 1940) and used to calculate specific activities.

Adenylate cyclase activity was assayed in a reaction mixture containing: 30 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM ATP, 1 mM GTP, 0.25 mM EGTA, 0.1% bovine serum albumin, 3 mM theophyline, 0.2 mg/ml pyruvate kinase and 5 mM phosphoenol pyruvate. The conversion of [α^{32}P]ATP to cyclic 3′,5′-[^{32}P]AMP was followed by the method of Ramachandran (1971) using 3′,5′-[^{14}C]AMP to enable correction for losses.

Sialyl and galactosyl transferases were assayed as described earlier (Riordan, Mitchell & Slavik, 1974) using asialofetuin and ovomucoid as acceptors, respectively. Saturating amounts of nucleotide-sugars and protein acceptors were employed.

Protein Assay

Proteins were determined by the fluorometric ('fluorescamine') method of Böhlen, Stein, Imai and Udenfriend (1973) using bovine serum albumin as standard.

^{125}I Wheat Germ Agglutinin Labelling

WGA was purified from crude wheat germ lipase (Sigma) according to Marchesi (1973) by affinity chromatography on ovomucoid-sepharose which was prepared by the method of Cuatrecases (1970). Labelling of WGA with ^{125}I was performed by adding 10 µl of 0.1 M NaH_2PO_4, pH 7.5, containing 200 µg of the purified WGA to 100 µl of the same buffer containing 1 mC $Na^{125}I$ (carrier-free). 2.0 µg of lactoperoxidase and 2 µl of 10 mM H_2O_2 were added. After a 20 min incubation at room temperature, the reaction

was terminated by adding 2.5 ml of ice-cold 0.05 M Tris-HCl, pH 7.5. The ^{125}I-WGA was isolated from this mixture using the same affinity chromatography procedure used to purify the native lectin. The pure ^{125}I-WGA had a specific activity of 1.2 μC/μg.

The lactoperoxidase used for the radio-iodination had been purified from whole milk by the procedure of Morrison and Hultquist (1963).

Labelling of the fibroblast surface before isolation of plasma membranes was performed using cells which had been washed extensively with PBS. Twenty-five μg (6×10^7 dpm) of ^{125}I-WGA in 10 ml of PBS was added to a roller bottle which was rotated for 30 min at 37 °C. The labelled cells were further washed two times with PBS before beginning the membrane isolation procedure. Aliquots were taken at various stages of the fractionation for determination of ^{125}I radioactivity by γ counting.

Results

Method One-Hypotonic Treatment

Membranes were isolated using cell strains from three donors with similar results for each. Representative results are presented. Phase-contrast microscopy first indicated that the suspension obtained by hypotonic swelling of cells followed by manual shaking of the roller bottles contained very few intact cells. Hence, this rapid and convenient procedure obviated the need for further disruptive procedures such as homogenization. Since our aim was to achieve a straightforward and efficient large scale method, it was decided to pellet nearly all the particulate material in this suspension by centrifugation at $27{,}000 \times g$ for 30 min rather than first spinning out nuclei with a lower speed spin. Evaluation of the membrane isolation by assays of marker enzymes, surface labels and electron microscopy is described below.

5′-Nucleotidase Distribution during Subcellular Fractionation

Although this enzyme has been widely used as a marker for plasma membranes (Solymon & Trams, 1972) on the basis of the histochemical demonstration of this localization in liver (Segal & Brenner, 1960), there have been suggestions that this may not be the case in some cultured cells (Wallach & Ullrey, 1962). Therefore, experiments were performed to determine the extent to which 5′-nucleotidase is an ecto-enzyme of cultured human fibroblasts. Greater than 93% of the total activity assayed in a homogenate of 5×10^5 cells prepared in the presence or absence of 0.5% Lubrol PX was accounted for by the activity at the surface of the intact cells prior to homogenization. No activity was

Table 1. Protein and 5′-nucleotidase in cell lysate and final fractions

Fraction	Protein		5′-Nucleotidase		
	mg	%	Specific	Total	%
lysate	771	100	2.0	1542	100
10/30	11	1.4	69.0	711	46.1
30/48	18	2.3	3.8	68	4.4
48/60	38	4.9	2.6	99	6.4

Assays were performed as described in Methods. The specific activities of 5′-nucleotidase are in units of μmoles Pi released per mg protein·hr and total activities, μmoles per hr.

released from the cells into the incubation medium during a period of 1 hr which is much longer than the time normally used to assay the enzyme. Hence, the only alternative to localization of the enzyme at the plasma membrane's external surface would require uptake of AMP by cells, internal hydrolysis and release of the product, adenosine. Since plasma membranes are virtually impermeable to nucleotides (Glynn, 1968), this pathway appears highly unlikely. On this basis 5′-nucleotidase is an ecto-enzyme of human skin fibroblasts.

Table 1 shows that on completion of the isolation procedure the '10/30' fraction had by far the greatest specific activity and accounted for approximately 46% of the total amount of the enzyme in the lysate. Significant but much smaller amounts of the enzyme were present in the '30/48' and '48/60' fractions. Hence the distribution of this ecto-enzyme provides strong evidence that a major portion of the plasma membrane is recovered in the '10/30' fraction.

ATPase and Adenylate Cyclase Distribution

As demonstrated by Table 2, Mg^{2+}-ATPase of highest specific activity was present in the '10/30' fraction, although a significant amount was also present in the '30/48' fraction. The $(Na^+ + K^+)Mg^{2+}$ATPase, considered as perhaps the best plasma membrane marker because of its function role in this location (Solymon & Trams, 1972) was found almost exclusively in the '10/30' fraction.

Both PGE- and F^--stimulated adenylate cyclase activities were greatest in the '10/30' fraction as was the basal activity of this enzyme

Table 2. ATPase activities in cell lysate and final fractions

Fraction	Mg^{2+}-ATPase			$(Na^+ + K^+)Mg^{2+}$-ATPase		
	Specific	Total	%	Specific	Total	%
lysate	0.5	239	100	0.3	223	100
10/30	3.9	40.3	11.8	1.8	86	38.6
30/48	2.2	16.6	7.1	0	0	0
48/60	0.5	5.6	2.4	0.4	6.8	3.1

Assays were as described in Methods and specific activities are in units of μmoles Pi released per mg protein·hr and total activities, μmoles per hr.

Table 3. Adenylate cyclase in final fractions

Fraction	Basal		$+PGE_1$		$+F^-$	
	Specific	Total	Specific	Total	Specific	Total
10/30	16.4	343.5	211.0	5034.0	105.0	2344.0
30/48	7.5	112.2	52.0	1190.0	24.0	550.0
48/60	11.1	198.7	22.5	402.0	35.9	643.0

Activities were assayed as described in Methods. Specific activities are in units of pmoles cAMP formed per mg protein·min and total activities, pmoles per min.

(Table 3). The basal activity, however, did not differ as greatly among the different fractions from the sucrose gradient, consistent with other suggestions that it may also be associated with intracellular fractions (Solymon & Trams, 1972).

Enzyme Markers of Other Organelles

In order to assess the degree of contamination of the plasma membrane-enriched fraction, enzymes which are markers of other subcellular fractions were assayed. Sialyl and galactosyl transferases in the '10/30' fraction were both enriched about twofold over their activities in the total cell lysate (Table 4). The specific activities in the '30/48' and '48/60' fractions were less than in the homogenate. Thus, while none of these fractions show any marked purification in these enzymes, the '10/30' fraction probably does contain some of the organelles (primarily Golgi) in which they reside *in situ*.

Table 4. Glycosyl transferases in cell lysate and final fractions

Fraction	Sialyl transferase			Galactosyl transferase		
	Specific	Total	%	Specific	Total	%
lysate	1.25	929.0	100	1.09	810.0	100
10/30	2.86	50.1	5.4	2.08	36.4	4.5
30/48	1.03	23.6	2.5	0.65	14.9	1.8
48/60	0.99	17.7	1.9	0.97	17.4	2.1

Activities were assayed with protein acceptors as described in Methods and were corrected for nonacceptor activity. Specific activities are in units of pmoles of sugar transferred per mg protein·min.

Table 5. Other marker enzymes in cell lysate and final fractions

Fraction	Succinate dehydrogenase			Glucose-6-phosphatase		
	Specific	Total	%	Specific	Total	%
lysate	0.83	619	100	0.13	61.3	100
10/30	0.44	6.7	1.2	0.24	1.3	2.1
30/48	0.60	13.8	2.2	0.19	1.5	2.4
48/60	0.08	1.5	0.2	0.29	3.1	5.0

Assays were as described in Methods. Specific activities are in units of μmoles of substrate converted per mg protein·hr and total activities, μmoles per hr.

The mitochondrial marker succinate dehydrogenase exhibited its highest specific activity in the '30/48' fraction although considerable activity was also measured in the '10/30' fraction (Table 5). All of the fractions from the sucrose gradient, however, are depleted in this enzyme since their specific activities are lower than that of the lysate. The total recovery of this enzyme is lower than expected.

Glucose-6-phosphatase activity was spread across each each of the fractions collected from the sucrose gradient with the highest specific activities in the '10/30' and '30/48' fractions (Table 5).

^{125}I *Wheat Germ Agglutinin Distribution*

The specific activities and recoveries at the various stages of fractionation after binding of ^{125}I-WGA to the surface of the cells are shown

Table 6. ^{125}I-wheat germ agglutinin in cell lysate and final fractions after binding to cell surface

Fraction	Specific binding	Total binding	%
lysate	2.5	2.00×10^6	100
10/30	7.2	0.35×10^6	17.5
30/48	2.9	0.09×10^6	4.5
48/60	5.0	0.10×10^6	5.0

Prior to disruption and fractionation cells were labelled as described in Methods. Specific binding is dpm/mg protein in each fraction and total binding, dpm.

in Table 6. Most of the lectin was associated with the total particulate fraction of the disrupted cells and after sucrose density centrifugation the greatest specific binding activity and recovery was in the '10/30' fraction. Although only small proportions of the total radioactively labelled lectin bound were recovered in the '30/48' and '40/60' fractions, the specific activities in these fractions were about 40 and 70%, respectively, of that in the '10/30' fraction.

Electron Microscopy

Fig. 3 shows electron micrographs of the three final fractions obtained from the sucrose gradient. The '10/30' fraction consists mainly of smooth vesicles ranging from a fraction of a μm to about 2 μm in diameter. A few flat plates less than a tenth of a μm in thickness and approximately 1 μm in length can also be seen. The presence of sialyl and galactosyl transferases of relatively high specific activity in the '10/30' fraction (Table 4) indicates the presence of constituents of the Golgi apparatus in this fraction. The flat plates observed here resemble the plates of the Golgi. Hence two types of evidence point to the presence of some Golgi material in this plasma membrane-enriched fraction. Aside from this, some small (< 0.2 μm) electron-dense material which is unidentifiable is present.

Most conspicuous in the '30/48' fraction (Fig. 3) is the large amount of rough microsomes, much of which appears as large ribosome-studded sheets of membrane. This contrasts the small ribosome covered spherical vesicles seen in classical rough microsome preparations from some cell types (Palade, 1959) and seems to indicate that these intracellular structures have not been reduced to their smallest stable units in our case. Some sheets and vesicles of smooth membrane are also present.

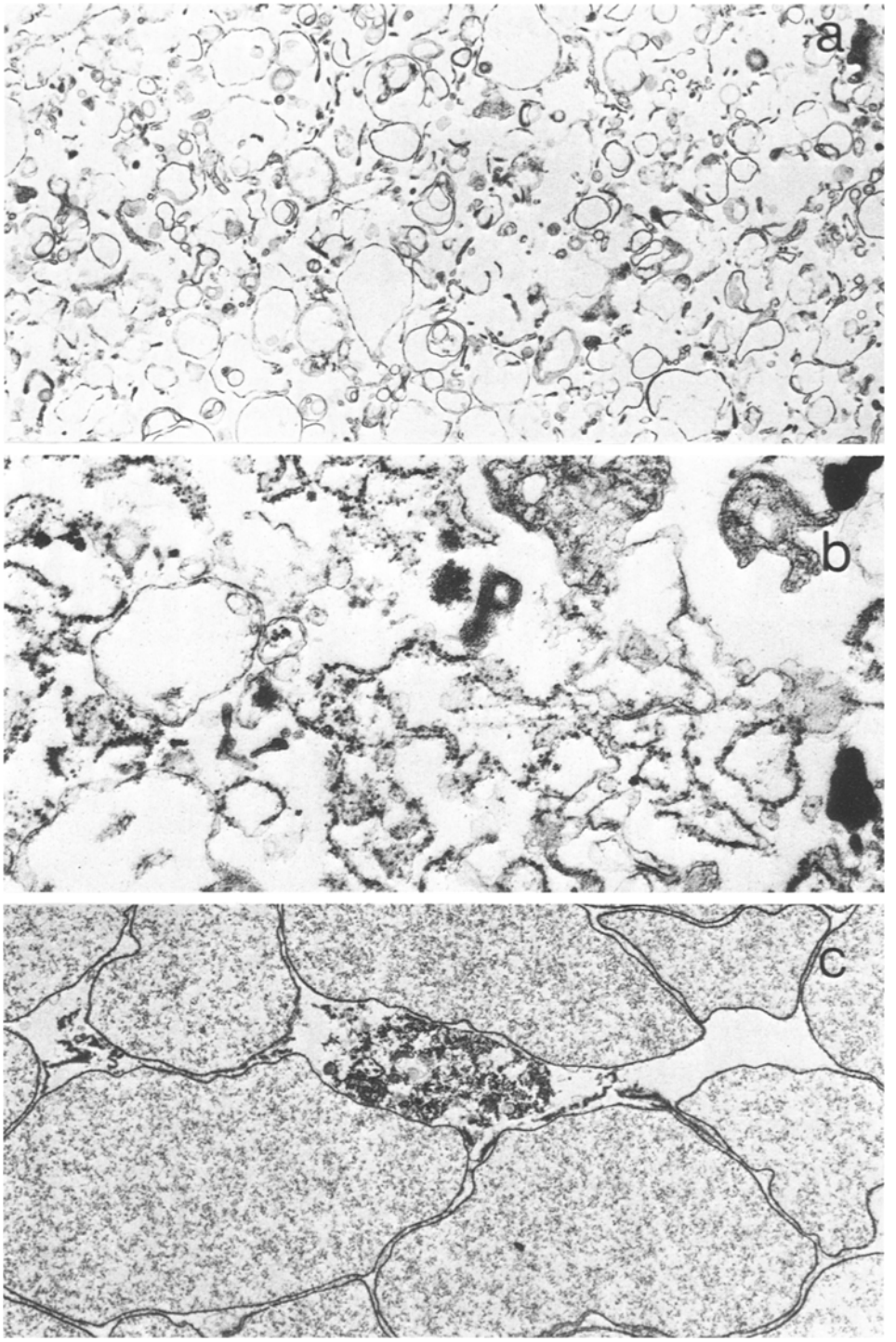

Fig. 3. Electron micrographs of thin sections through pellets of the materials obtained at the interfaces of the sucrose gradient used in Method One. (*a*) '10/30' fraction (mag. 11,000×); (*b*) '30/48' fraction (mag. 21,000×); (*c*) '48/60' fraction (mag. 5,000×)

The '48/60' fraction consists primarily of intact nuclei; a few appear fragmented. Significant amounts of membranous material appear around the periphery of each nucleus. It is likely that these membranes are carried into this high density fraction by virtue of their adherence to the nuclear surface. In fact, if a chelating agent such as EDTA is not added to the disrupted cells prior to fractionation, large amounts of membrane, likely of both cell surface and intracellular origin stick to nuclei and cannot be easily separated from them. This process is augmented if divalent cations are added before or after cell disruption. The addition of EDTA, after disruption rather than before is an important feature of our procedure. When EDTA was present in the hypotonic medium (1 mM $NaHCO_3$) used for disruption, a large number of nuclei were damaged with release of chromatin which, due to its high viscosity, interfered with the subsequent separation of subcellular fractions.

Subfractionation of '10/30' Fraction

As judged by the criteria just described in detail the '10/30' fraction qualifies as a plasma membrane-enriched fraction. However, if the material removed from the '10/30' interface was pelleted in two steps, lighter and heavier subfractions could be obtained. The lighter fraction was pelleted by centrifugation at $27{,}000 \times g$ for 20 min, the heavier by further spinning the supernatant at $76{,}000 \times g$ for 20 min. Morphologically the lighter fraction contained more of the smaller range of vesicles (mainly < 0.5 μm) while the heavier fraction contained a greater proportion of larger vesicles (0.5–2.0 μm). A considerable quantity of fibrous material which could not be definitively identified was also present in the lighter fraction but totally absent from the heavier. The lighter fraction had a lower 5′-nucleotidase specific activity than the heavier (e.g., 43 and 72 μmoles/mg prot·hr, respectively), a lower Mg^{2+}-ATPase (e.g., 2.7 and 5.1 μmoles/mg prot·hr, respectively), and a lower PGE_1-activated adenylate cyclase (125 and 195 pmoles/mg prot·min, respectively). The light fraction exhibited a higher glucose-6-phosphate specific activity than any of the other fractions indicating that a significant proportion of the vesicles present may be derived from the smooth endoplasmic reticulum. However, the cell surface membrane also has a highly active nonspecific phosphatase which does hydrolyze glucose-6-phosphate (Slavik, Kartner & Riordan, 1977). Therefore, smooth microsomes are not necessarily responsible for the activity observed.

Table 7. Protein and 5′-nucleotidase in cell homogenate and final fractions from Method Two

Fraction	Protein		5′-Nucleotidase		
	mg	%	Specific	Total	%
homogenate	162	100	3.8	616.0	100
10/25	0.9	0.5	22.0	19.4	3.2
25/35	2.4	1.5	88.0	211.0	34.0
35/45	2.0	1.2	12.0	24.0	4.0
45/55	1.9	1.2	5.4	10.3	1.7
pellet 1	2.5	1.5	2.9	7.3	1.2
25/45	0.5	0.3	9.8	4.9	0.8
pellet 2	0.3	0.2	2.8	0.7	0.1

Assay methods and units as in Table 1.

Method Two-Isotonic Homogenization

The procedure described above was considered to be an efficient and straightforward means of obtaining a highly enriched plasma membrane fraction from large quantities (10^9 cells) of cultured human fibroblasts. Time-consuming steps which were avoided included scraping of cells from the roller bottle surfaces, homogenization and a low speed centrifugation step to remove nuclei. In order to ascertain the influence of deleting or replacing these steps, a second procedure (Fig. 2) which included them as well as a more complex sucrose gradient was attempted. Since 5′-nucleotidase was demonstrated to be almost totally an ectoenzyme of the plasma membrane of these cells, it was relied upon along with electron microscopy as a monitor of this fractionation.

5′-Nucleotidase Distribution

Table 7 shows the results of a typical membrane purification using this method. By far the highest 5′-nucleotidase specific activity and recovery appeared in the '25/35' fraction. The specific activity (88 μmoles/mg prot·hr) was slightly higher than that of the '10/30' fraction from the first method but the yield (34%) was less. The specific activity of the '10/25' fraction was considerably less (22 μmoles/mg prot·hr) and the total amount of activity recovered in this fraction was also low. The '35/45' fraction also exhibited some activity and the '45/55' fraction a lesser amount. The slight activity in the pellet presumably reflects

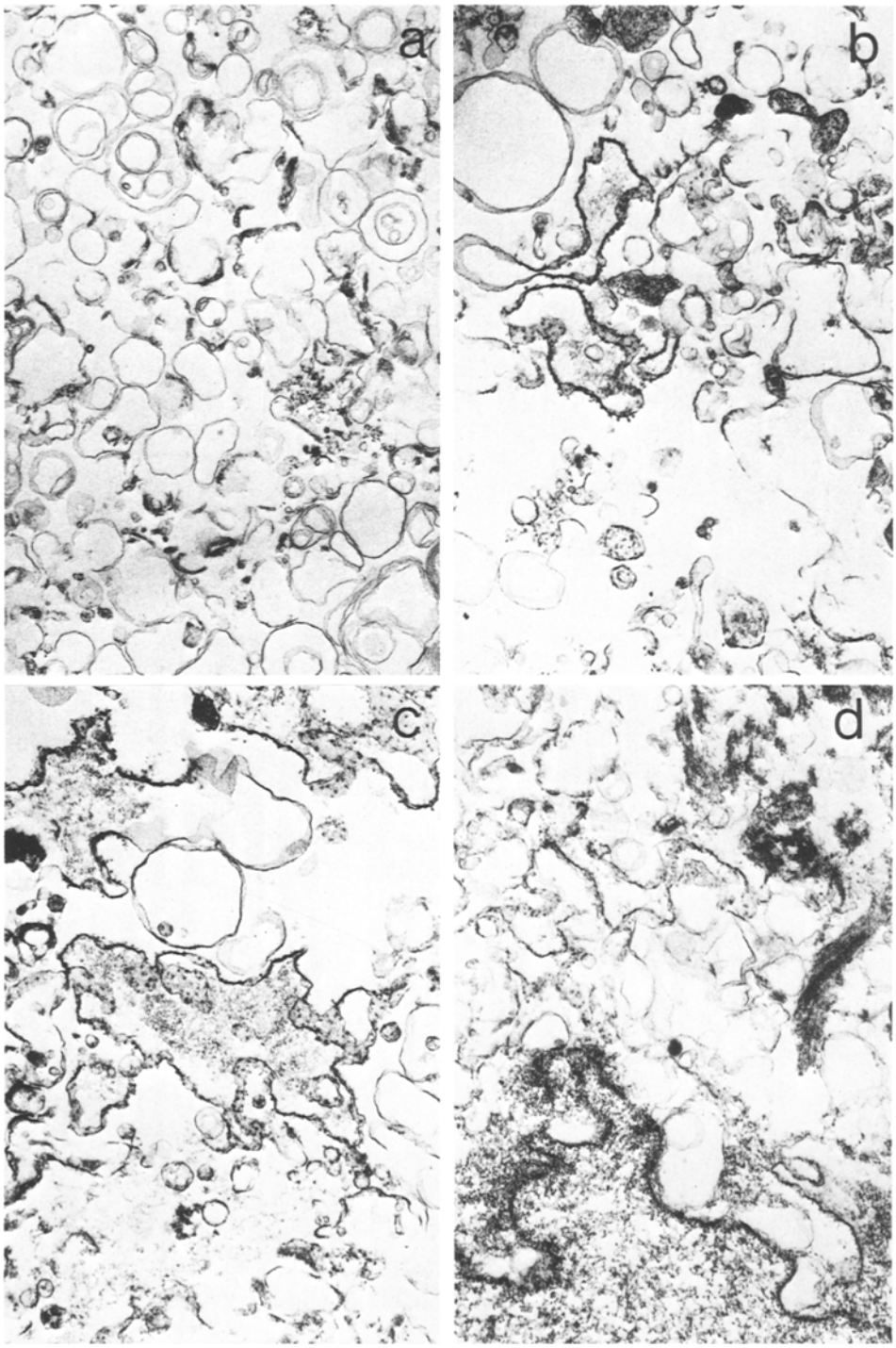

Fig. 4*a–d*

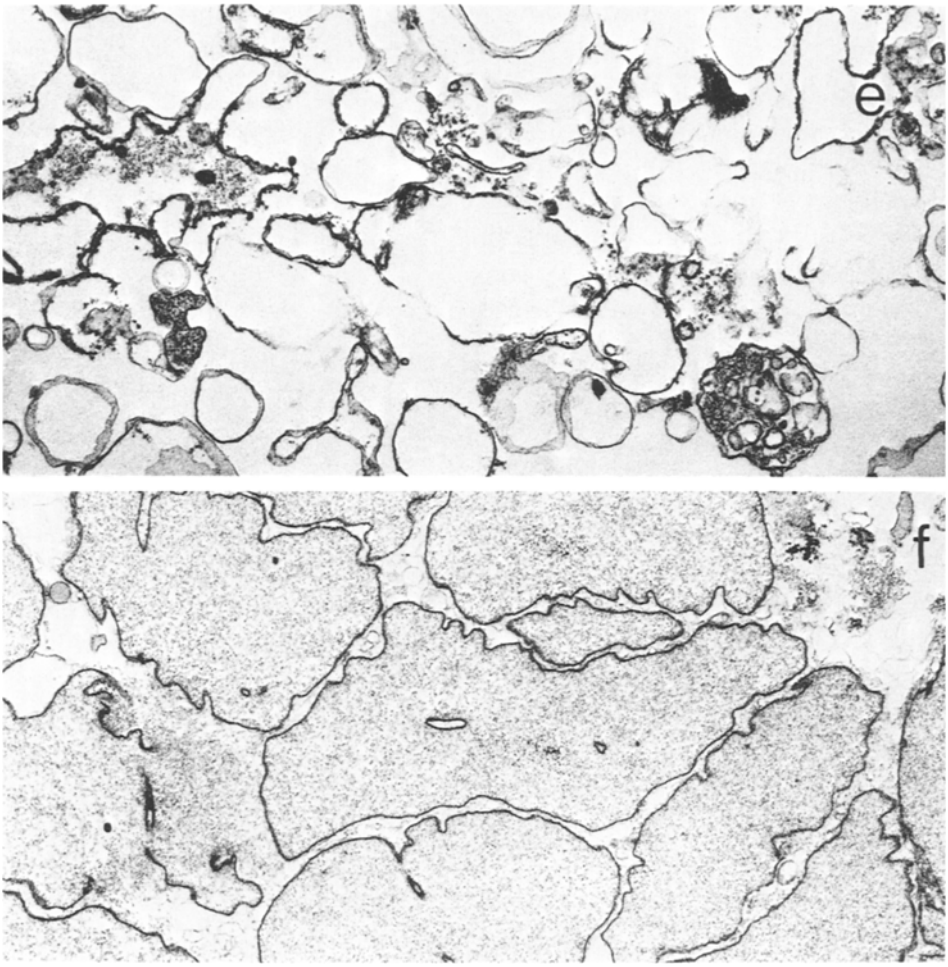

Fig. 4. Sections through pellets of fractions obtained in Method Two. (*a*) '10/25' fraction (mag. 15,000×); (*b*) '25/35' fraction (mag. 21,000×); (*c*) '35/45' fraction (mag. 21,000×); (*d*) '45/55' fraction (mag. 15,000×); (*e*) '25/45' fraction from gradient centrifugation of low speed (700×*g*) nuclear pellet (mag. 21,000×); (*f*) 'pellet' from gradient centrifugation of nuclear pellet (mag. 21,000×)

that which resides in membrane fragments adhering strongly to intact nuclei. A small portion of the enzyme is present in the '25/45' fraction after gradient centrifugation of the nuclear pellet.

Electron microscopy

Fig. 4*a* shows that the '10/25' consists primarily of smooth vesicles of similar size to those seen in the '10/30' fractions of the first method.

Relatively fewer flat plates, thought to be derived from Golgi, were present. These structures are known to be especially sensitive to the shearing forces of homogenization (Sturgess, Katona & Moscarello, 1973). The '25/35' (Fig. 4*b*) fraction contains many similar vesicles and, in addition, large sheets of membrane, some with associated ribosomes. Rough microsomes appear to be the major constituent of the '35/45' (Fig. 4*c*) fraction which appears similar to the '30/48' fractions of the hypotonic method. The '45/55' fraction contains some intact and some broken nuclei, nuclear membranes which attached chromatin and electron-dense debris (Fig. 4*d*).

In this procedure the nuclear pellet (sedimented at $700 \times g$) was observed by phase-contrast microscopy to contain considerable membranous material. Thus, a second density gradient centrifugation utilizing only 10, 25 and 45% sucrose was used to resolve these membranes from nuclei. Nothing banded at the '10/25' interface presumably because no material of this size and shape would have been sedimented at $700 \times g$. The electron micrograph of the '25/45' fraction shows mainly sheets and vesicles with and without attached ribosomes (Fig. 4*e*). Hence, this fraction was apparently similar to the sum of the '25/35' and '35/45' fractions from the first density gradient centrifugation. Trapping in the nuclear pellet, however, would result in a decreased recovery of these structures if only the supernatant of the low speed centrifugation were subjected to density gradient centrifugation. The pellet below the 45% sucrose (Fig. 4*f*) contained intact nuclei and some attached electron-dense structures similar to those in the '45/55' fraction from the first gradient.

Discussion

Plasma membrane-enriched fractions have been prepared from cultured fibroblasts derived from human skin biopsies. To avoid degradation of cell surface components, the cells were harvested by mechanically removing them from the substratum rather than using protease treatment. Disruption of the harvested cells under both hypotonic and isotonic conditions resulted in the vesiculation of membranes which occurs with most cell types when the cell surface has not been stabilized or fixed (Wallach & Lin, 1973). HeLa cells are apparently an exception in this respect since large broken plasma membrane ghosts have been obtained after disruption by homogenization in hypotonic medium although no fixatives were used (Bosmann *et al.*, 1968). The membrane vesicles exhibit-

ing the highest specific activities and recoveries of plasma membrane markers banded on top of sucrose of a density of 1.12 after density gradient centrifugation.

The purification factors and recoveries of the plasma membrane markers used compared very favorably with those obtained by other investigators using other cultured cells (Brunette & Till, 1971) including fibroblasts from different animal and tissue sources (Gahmberg & Simons, 1970; Perdue & Sneider, 1970). 5′-Nucleotidase which was shown to be an ecto-enzyme of these cells was enriched approximately 20-fold compared to the cell homogenate. Nearly half of the total amount of the enzyme was recovered in the plasma membrane-enriched fraction. Two true plasma membrane markers, $(Na^+ + K^+)Mg^{2+}$-ATPase and PGE- and F^--activated adenylate cyclase exhibited the highest specific activities in the same fraction. However, accurate quantitative determination of enrichment factors and recoveries for these two enzymes was not possible because of the difficulties in accurately determining the specific activities of the dilute hypotonic homogenates. An exogenous marker, ^{125}I-WGA was recovered primarily in the plasma membrane fraction which also had the highest specific binding activity. Although the recovery was small, the material fractionating at the interface between 48 and 60% sucrose had a specific binding activity about 70% as great as that in the plasma membrane fraction. This is interpreted to indicate that at least some of the membranous material seen adhering to the surface of intact nuclei in this fraction were derived from the cell surface. However, the fact that binding of lectins such as WGA to their receptors is readily reversible even at low temperature in the presence (but not in the absence) of haptene inhibitors of binding means that some exchange of ^{125}I-WGA initially bound to the plasma membrane with oligosaccharides of intracellular membranes may occur during the isolation procedures. This could account for the relatively higher specific activity of this marker than that of the intrinsic enzyme markers of the plasma membrane in the '30/48' and '48/60' fractions. There is now ample evidence for lectin receptors in intracellular membranes (Young, Moscarello & Riordan, 1976). It is also possible that some of the ^{125}I-WGA was internalized by the cells during binding. Chang, Bennett and Cuatrecasas (1975) demonstrated that ^{125}I-WGA fractionated more exclusively to the plasma membrane fractions of fat cells, lymphocytes and liver when binding was performed for 5 or 10 min at 4 °C. However, in our experiments 0.1 M N-acetyl-D-glucosamine displaced 87% of the ^{125}I-WGA which had bound in 1 hr at 37 °C. Therefore, it is unlikely that

more than 13% of the labelled lectin had entered the cells under these conditions. Certainly, after binding for only $^1/_2$ hr prior to subcellular fractionation less than this amount would have been internalized.

Electron micrographs of thin sections of the preparations indicated the presence of flat plates, likely of Golgi origin, and the absence of rough microsomes and other identifiable organelles. The presence of smooth microsomes could not be ascertained in this way but considerable glucose-6-phosphatase activity makes it likely that some of the vesicles are of this origin. The lack of complete resolution of these from plasma membrane-derived vesicles is perhaps the most serious drawback of this type of procedure (Wallach & Kamat, 1964; Wallach & Lin, 1973). Nevertheless, when weighed against the denaturing and crosslinking effects of the heavy metal or aldehyde treatments necessary for the preparation of ghosts, the method appears to be a reasonable alternative, especially for investigations of functional aspects of the plasma membranes of these cells. Furthermore, when cells grown in monolayers are subjected to fixation, only the portion of the plasma membrane of the upper free half of the cell are obtained as large fragments (Barland & Schroeder, 1970).

The decision to isolate the plasma membrane as vesicles rather than ghosts led us to employ density gradient centrifugation for resolution from other organelles rather than another method such as partitioning in an aqueous polymer system. In fact, the dextran-polyethylene glycol system as utilized by Brunette and Till (1971) was tried but, as expected, little material was obtained at the interface between the two mixed polymer phases. However, when the centrifugal force applied was increased from 15,000 to 50,000 $\times g$ utilizing an ultracentrifuge, a reasonable yield of membrane vesicles was obtained at the interface between the phases. In three such preparations specific activities of 5′-nucleotidase ranged from 43 to 71 μmoles per mg · hr. From 57 to 69% of the total homogenate activity was recovered in these fractions. Thus, it seems that the surface properties responsible for the partitioning of the membranes are exhibited by the vesicles as well as by the larger ghosts. However, the smaller size of the vesicles requires the application of higher centrifugal forces and thus eliminates one of the major advantages of this procedure, viz. the short-term, low-speed centrifugation.

Methods of fractionation other than either density gradient centrifugation or partitioning in aqueous polymer systems could also be applied after the harvesting and disrupting of the cells by the methods described. For example, the demonstration that 5′-nucleotidase is almost exclusively

an ecto-enzyme of these cells is suggestive that AMP or an analog thereof, attached to an insoluble support such as agarose may be useful for selective binding of plasma membrane-derived vesicles. In fact, selectivity might be even greater than desired since only vesicles with a 'right-side-out' orientation would be expected to bind. Nevertheless, this method or others such as the "affinity density perturbation" approach introduced by Wallach and co-workers (1972) and utilized by others (Lim, Molday, Huang & Yen, 1975) may prove superior to resolution simply on the basis of density, size and shape on our sucrose gradients. However, these methods are not without their limitations and disadvantages such as the nonspecific association of various subcellular constituents with the perturbant. It is also possible that a somewhat improved resolution of vesicles from different sources might be achieved using ficol gradients after manipulation of divalent cation concentrations according to Kamat and Wallach (1965).

It is worth making note of the fact that the membrane vesicles obtained in the two procedures were similar despite the fact that in both cases disruption was extensive, our only limiting criterion being that disruption should cease prior to nuclear damage. Milder forms of disruption with the aim of obtaining larger plasma membrane fragments at the expense of leaving greater proportions of unbroken cells were not employed. Attempts at this approach with these cells produced extensive variability in both the quantity and quality of membranes obtained. In contrast, the reproducibility in either of the two methods arrived at was high, probably because the vesicles formed are close to the smallest stable subunits of the membrane. In the first method these are produced by osmotic vesiculation and mechanical shaking, and in the second case, by pinching off from the membrane due to the shearing forces of the homogenization. This reproducibility is especially desirable because of our aim of comparing membranes from cells derived from patients with genetic defects with those from matched normals.

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References

Allen, R.J.L. 1940. The estimation of phosphorus. *Biochem. J.* **34**:858

Atkinson, P.H. 1973. HeLa cell plasma membranes. *In:* Methods in Cell Biology. D.M. Prescott, editor. Vol. VII, p. 157. Academic Press Inc., New York and London

Barland, P., Schroeder, E.A. 1970. A new rapid method for the isolation of surface membranes from tissue culture cells. *J. Cell Biol.* **45**:662

Bingham, R.W., Burke, D.C. 1972. Isolation of plasma membrane and endoplasmic reticulum fragments from chick embryo fibroblasts. *Biochim. Biophys. Acta* **274**:348

Böhlen, P., Stein, S., Imai, K., Udenfriend, S. 1973. Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* **155**:213

Bolis, L., Hoffman, J.F., Leaf, A., editors. 1976. Membranes and Disease. Raven Press, New York

Bosmann, H.B., Hagopian, A., Eylar, E.H. 1968. Cellular membranes: The isolation and characterization of the plasma and smooth membranes of HeLa cells. *Arch. Biochem. Biophys.* **128**:51

Brown, M.S., Goldstein, J.L. 1976. Receptor-mediated control of cholesterol metabolism. *Science* **191**:150

Brunette, D.M., Till, J.E. 1971. A rapid method for the isolation of L-cell surface membranes using an aqueous two-phase polymer system. *J. Membrane Biol.* **5**:215

Chang, K.J., Bennett, V., Cuatrecasas, P. 1975. Membrane receptors as general markers for plasma membrane isolation procedures. *J. Biol. Chem.* **250**:488

Cuatrecasas, P. 1970. Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. *J. Biol. Chem.* **245**:3059

Engelhardt, R. 1976. Impaired reassemblance of red blood cell membrane components in hereditary spherocytosis. *In:* Membranes and Disease. L. Bolis, J.F. Hoffman, and A. Leaf, editors. p. 75. Raven Press, New York

Gahmberg, C.G., Simons, K. 1970. Isolation of plasma membrane fragments from BHK21 cells. *Acta Pathol. Microbiol. Scand.* **B78**:176

Glynn, I.M. 1968. Membrane ATPase and cation transport. *Br. Med. Bull.* **24**:165

Goldstein, J.L., Brown, M.S. 1976. LDL pathway in human fibroblasts: A receptor mediated mechanism for the regulation of cholesterol metabolism. *In:* Current Topics in Cellular Regulation. B.L. Horecker and E.R. Statman, editors. Vol. 11, p. 147. Academic Press Inc., New York (*in press*)

Goldstein, S., Littlefield, J.W. 1969. Effect of insulin on the conversion of glucose-C-14 to C-14-O_2 by normal and diabetic fibroblasts in culture. *Diabetes* **18**:545

Juliano, R.J., Gagalang, E. 1975. The isolation and characterization of the plasma membrane of cultured chinese hamster ovary cells. *Can. J. Biochem.* **53**:914

Kamat, V.B., Wallach, D.F.H. 1965. Separation and partial purification of plasma membrane fragments from Ehrlich ascites carcinoma microsomes. *Science* **148**:1343

Kartner, N., Alon, N., Swift, M., Buchwald, M., Riordan, J.R. 1976. Isolation of plasma membranes of cultured skin fibroblasts from children with cystic fibrosis. *Proc. Can. Fed. Biol. Soc.* **19**:53

Lesko, L., Donlon, M., Marinetti, G.V., Hare, J.D. 1973. A rapid method for the isolation of rat liver plasma membranes using an aqueous two-phase polymer system. *Biochim. Biophys. Acta* **311**:173

Lim, R.W., Molday, R.S., Huang, H.V., Yen, S.P.J. 1975. Application of latex microspheres in the isolation of plasma membranes. Affinity density perturbation of erythrocyte membranes. *Biochim. Biophys. Acta* **394**:377

Marchesi, V.T. 1973. Wheat germ (*Triticum vulgaris*) agglutinin. *Meth. Enzymol.* **28**:354

Morrison, M., Hultquist, D.E. 1963. Lactoperoxidase. II. Isolation. *J. Biol. Chem.* **238**:2847

Palade, G.E. 1959. Functional changes in the structure of cell components. *In:* Subcellular Particles. T. Hayashi, editor. p. 64. Ronald Press, New York

Perdue, J.F., Sneider, J. 1970. The isolation and characterization of the plasma membrane from chick embryo fibroblasts. *Biochim. Biophys. Acta* **196**:141

Ramachandran, J. 1971. A new simple method for separation of adenosine 3′5′-cyclic monophosphate from other nucleotides and its use in the assay of adenylate cyclase. *Anal. Biochem.* **43**:227

Riordan, J.R., Mitchell, L., Slavik, M. 1974. The binding of asialo-glycoprotein to isolated Golgi apparatus. *Biochem. Biophys. Res. Commun.* **59**:1373

Roses, A.D., Roses, M.J., Miller, J.E., Hull, K.L., Jr., Appel, S.H. 1976. Carrier detection in Duchenne muscular dystrophy. *N. Engl. J. Med.* **294**:193

Schimmel, S.D., Kent, C., Bischoft, R., Vagelos, P.R. 1973. Plasma membranes from cultured muscle cells: Isolation procedure and preparation of putative plasma membrane marker enzymes. *Proc. Nat. Acad. Sci. USA* **70**:3195

Segal, H.L., Brenner, B.M. 1960. 5′-Nucleotidase of rat liver microsomes. *J. Biol. Chem.* **235**:471

Slavik, M., Kartner, N., Riordan, J.R. 1977. Lectin-induced inhibition of plasma membrane 5′-nucleotidase: Sensitivity of purified enzyme. *Biochem. Biophys. Res. Commun.* **75**:342

Solymon, A., Trams, E.G. 1972. Enzyme markers in characterization of isolated plasma membranes. *Enzyme* **13**:329

Stanners, C.F., Eliceiri, G.L., Green, H. 1971. Two types of ribosome in mouse-hamster hybrid cells. *Nature, New Biol.* **230**:52

Sturgess, J.M., Katona, E., Moscarello, M.A. 1973. The Golgi complex. I. Isolation and ultrastructure in normal rat liver. *J. Membrane Biol.* **12**:367

Sullivan, A.K., Jerry, L.M. 1974. Application of a two-phase polymer system to the isolation of lymphocyte plasma membranes. *Can. Fed. Biol. Soc.* **17**:113

Suran, A.A. 1973. A simple microradioisotopic assay for 5′-nucleotidase activity. *Anal. Biochem.* **55**:593

Wallach, D.F.H., Kamat, V.B. 1964. Plasma and cytoplasmic membrane fragments from Ehrlich ascites carcinoma. *Proc. Nat. Acad. Sci. USA* **52**:721

Wallach, D.F.H., Kranz, B., Ferber, E., Fischer, H. 1972. Affinity density perturbation: A new fractionation principle and its illustration in a membrane separation. *FEBS Lett.* **21**:29

Wallach, D.F.H., Lin, P.S. 1973. A critical evaluation of plasma membrane fractionation. *Biochim. Biophys. Acta* **300**:211

Wallach, D.F.H., Ullrey, D. 1962. The hydrolysis of ATP and related nucleotides by Ehrlich ascites carcinoma cells. *Cancer Res.* **22**:228

Warren, L., Glick, M.C., Nass, M.K. 1966. Membranes of animal cells. I. Methods of isolation of the surface membrane. *J. Cell. Physiol.* **68**:269

Young, M.E.M., Moscarello, M.A., Riordan, J.R. 1976. Concanavalin A binding to membranes of the Golgi apparatus and resultant modification of galactosyl transferase activity. *J. Biol. Chem.* **251**:5860