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IMPROVED METHOD FOR THE ISOLATION OF RAT LIVER PLASMA MEMBRANE

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SUMMARY

An improved method for the isolation of plasma membrane from rat liver is presented.

Gentle homogenization of perfused livers in buffered isotonic KCl, followed by direct flotation of a low-speed nuclear pellet through a discontinuous sucrose density gradient results in a 32 % yield, and 25-fold enrichment for the plasma membrane marker, phosphodiesterase I, in a crude plasma membrane fraction. This fraction contains less than 1 % of the mitochondria, and endoplasmic reticulum present in the original homogenate, but is more heavily contaminated with lysosomes and Golgi membrane.

Vigorous mechanical disruption of this material, followed by a second discontinuous sucrose density gradient, gives a light plasma membrane fraction with an 80-fold purification and 20 % yield of phosphodiesterase I over the original homogete (with further reduction of contaminants).

INTRODUCTION

Since the original publication by Neville [1], a number of methods have been described for isolating plasma membrane fragments from tissues such as liver (for reviews, see refs 2–4). However, problems of yield, purity and reproducibility encountered with several methods prompted us to develop a somewhat different procedure, which is the subject of this communication. In our hands, this preparation provides material with yield and purity more suitable for isolation of individual plasma membrane proteins.

MATERIALS

All sucrose solutions are given as % w/w. *p*-Nitrophenyl 5′thymidylate was purchased from Calbiochem, San Diego, Calif. Cytochrome *c*, NADPH, AMP,

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MOPES, morpholinopropane-sulfonic acid.

p-nitrophenyl phosphate, tris(hydroxymethyl)-aminomethane (Tris), *N*-2-hydroxylethylpiperazine-*N'*-2'ethanesulfonic acid (HEPES), dithiothreitol and *p*-iodonitrotetrazolium violet were purchased from Sigma Chemical Company, St. Louis, Mo. UDP[U-^{14}C]galactose, 81 Ci/mol, was purchased from New England Nuclear Corporation, Boston, Mass. Osmium tetroxide was purchased from Ventron, Alpha Products, Beverly, Mass. Copper grids, 400 mesh, were obtained from Ted Pella Company, Tustin, Calif. Glutaraldehyde, 50 % biological grade, acrylamide and *N,N*-methylenebisacrylamide were purchased from Polyscience, Inc., Warrington, Pa. Sodium dodecyl sulfate was obtained from BDH Chemicals, Poole, England. Rabbit muscle actin was the generous gift of Dr. Michael P. Sheetz.

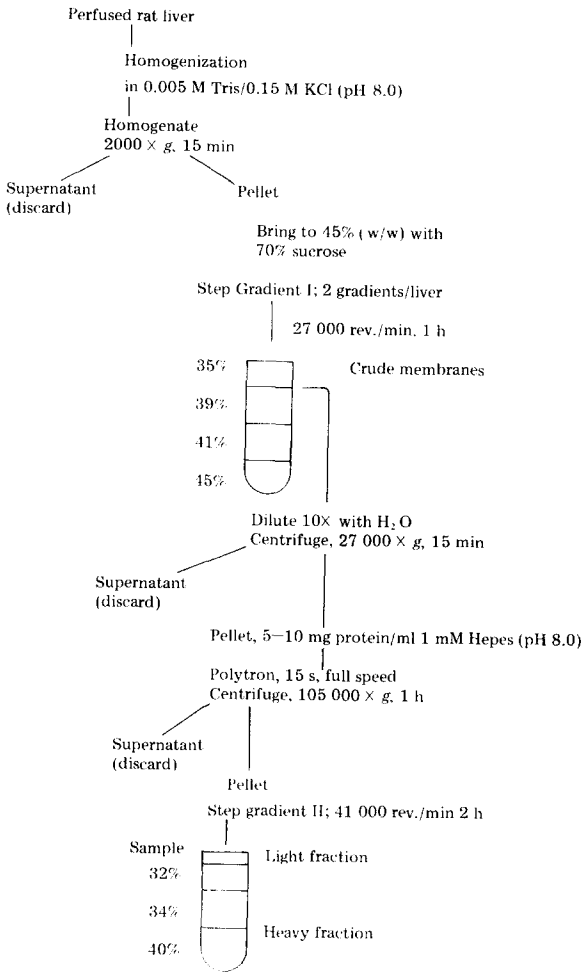
METHODS

Liver fractionation

Female albino rats of the Sprague-Dawley strain were fed ad libitum. Animals were anesthesized with ether, and the livers perfused through the hepatic portal vein with 0.85 % (w/v) saline at 37 °C. Livers were excised and washed in 0.005 M Tris/0.15 M KCl, pH 8.0 (Buffer A), at 4 °C, minced in 20–25 ml of buffer A in a Waring Blender, operating at full speed for 7 s, followed by homogenization with 25–30 strokes in a glass Dounce homogenizer fitted with a loose pestle. Cell breakage was monitored by light microscopy using Trypan Blue stain; homogenization was considered complete when only 1–2 intact whole cells were visible in a microscope field. After filtration through two layers of coarse gauze, the resulting material was designated as crude homogenate.

The homogenate was further fractionated as shown in Scheme 1. The crude homogenate was centrifuged at 2000 $\times g$ for 15 min in a Sorvall SS-34 rotor. This low speed pellet was adjusted to a final concentration of 45 % ($d = 1.2025$) with 70 % sucrose, and the pH was adjusted to 8.0 with 1 M NaOH. This material (7–9 ml) was transferred to a SW27 centrifuge tube, and carefully overlayered with 14 ml of 41 %, 14 ml of 39 % and 5 ml of 35 % sucrose in water, pH 8.0. Tubes were centrifuged at 27 000 rev./min for 1 h at 4 °C. A crude, plasma membrane-enriched fraction, which floated at the 35–39 % sucrose interface, was collected by aspiration and diluted about 10-fold with cold H_2O, and the membranes pelleted by centrifugation at 27 000 $\times g$ for 15 min in the Sorvall SS-34. Pellets were resuspended to about 5 mg protein/ml by gentle homogenization with a loose Dounce homogenizer.

Crude membranes were subfractionated by treating the suspension with a Polytron (Brinkman Instruments, Westbury, N.Y.) operating at full speed for 15 s with ice cooling. After centrifugation at 40 000 rev./min for 1 h, the resulting membrane pellet was resuspended in 1 mM HEPES buffer, pH 8.0, (Buffer B) to about 10 mg protein/ml, and layered onto a second discontinuous sucrose density gradient consisting of 3 ml of 32 % ,4 ml of 34 % and 4 ml of 40 % sucrose in water, pH 8.0. Centrifugation for 2 h at 41 000 rev./min in a Beckman SW41 gave a light fraction at the sample buffer-32 % sucrose interface, and a heavy fraction at the 34–40 % interface. These bands were collected by aspiration, resuspended with a loose Dounce homogenizer, and used directly for determination of protein and enzyme activities. When required membrane fractions were recovered from sucrose solutions by dilution with



Scheme 1. Isolation of rat liver plasma membrane.

water and centrifugation at 105 000 × *g* for 1 h, the resulting pellets were resuspended to 1 mg protein/ml in Buffer B.

Golgi membranes were prepared as described by Ehrenreich et al. [5].

Enzyme assays

Phosphodiesterase I was assayed in a total volume of 0.2 ml containing 2.0 mM *p*-nitrophenyl 5'-thymidylate, 0.1 M glycine buffer (pH 9.6) and 1 mg/ml Triton X-100. The appearance of *p*-nitrophenol was followed at 400 nm on a Gilford recording spectrophotometer, using 14.0 as the millimolar extinction coefficient.

NADPH-cytochrome c oxidoreductase was assayed in a total volume of 0.22 ml containing 0.09 M potassium phosphate buffer (pH 7.6), 4.5 mM ethylenediaminetetraacetic acid (EDTA), 9 mM potassium cyanide, 1 mg/ml Triton X-100, 0.01 mM cytochrome *c*, and 0.1 mM NADPH. The appearance of reduced cytochrome *c* was followed spectrophotometrically at 550 nm on a Gilford recording spectrophotometer.

UDPgalactose-N-acetylglucosamine galactosyltransferase was assayed at 37 °C in a total volume of 0.025 ml containing 0.06 M MOPES buffer (pH 6.0) 0.048 M $MnCl_2$; 0.02 M dithiothreitol, 0.4 mM UDP[U-^{14}C]galactose (5984 dpm/nmol), 10 mg desialyted degalactosylated rabbit fetuin [6] and 0.24 % Triton X-100. After 20 min, the reaction was stopped by the addition of 0.5 ml 0.01 M EDTA, followed by 2.0 ml of 10 % trichloroacetic acid. The protein precipitate was recovered by filtration onto 2.4 cm GF/A glass fibre papers, and washed with another ml of trichloroacetic acid, followed by two washes of 2 ml of 95 % ethanol. The filters were dried and counted in 2.0 ml of toluene scintillation fluid on a Packard TriCarb. Samples without enzyme, averaging about twice the background, served as controls for nonspecific label incorporation. No endogenous receptor activity was detectable in the absence of the exogenous acceptor.

Acid phosphatase was assayed [7] at 37 °C in a total volume of 0.2 ml containing 0.2 M citrate/NaOH buffer (pH 5.0) and 0.01 M sodium *p*-nitrophenyl phosphate. The reaction was stopped after 10 min by addition of 2.8 ml of 0.1 M NaOH and the appearance of *p*-nitrophenol was determined at 400 mn. Samples without enzyme served as controls, and these values were subtracted from experimental values. *Succinate dehydrogenase* was assayed [8] at 37 °C in a total volume of 0.3 ml containing 0.04 M potassium phosphate buffer (pH 7.5), 0.055 M sodium succinate and 9.2 % *p*-iodonitrotetrazolium violet. The reaction was stopped after 10 min by the addition of an equal volume of 10 % (w/v) perchloric acid, followed by 1.0 ml of ethyl acetate. The mixture was vigorously mixed, centrifuged, and the organic layer was read at 470 nm. A millimolar extinction of 20.0 for the product formazan was used to calculate enzyme activity.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out on 11 % (w/v) gels in the borate/Tris/sodium dodecyl sulfate buffer system of Neville [9]. Prior to electrophoresis, samples (5–75 μg) of proteins were heated at 80 °C in a solution containing 0.09 M dithiothreitol, 1 % sodium dodecyl sulfate and 0.009 M sodium carbonate. Cylindrical gels were run at 1.5 mA per gel, and 2.0 mm slab gels at 20 mA. Proteins on gels were fixed overnight with acetic acid/isopropanol/H_2O (2 : 5 : 13, v/v) [10], and stained with Coomassie Blue for 1 h, prior to diffusion destaining with acetic acid/methanol/H_2O (3 : 2 : 35, v/v).

Membrane fractions were extracted as described by Brown and Elovson [11], and cholesterol determined with the method of Rudel and Morris [12].

Protein was determined by the method of Lowry et al. [13] using bovine serum albumin as a standard. When sucrose was present in samples, protein was precipitated in the presence of 5 mg deoxycholate by the addition of trichloroacetic acid to a final concentration of 10 %. Samples were centrifuged, and protein was determined after solubilization with 0.1 M NaOH, by comparison to a protein standard prepared in a similar manner.

Membrane preparations to be analyzed for nucleic acid were extracted by the Schmidt-Thannhauser procedure as modified by Fleck and Munro [14]. DNA was determined by a modification [15] of the procedure of Giles and Meyers [16], using calf thymus DNA as a standard; diphenylamine was purified by recrystallization from hexane [17]. RNA was estimated by the orcinol method using the modification of Fleck and Munro [14], with yeast RNA as a standard. Orcinol was purified by recrystallization from benzene [17].

Electron microscopy

Cell fractions for electron microscopy were pelleted by centrifugation and fixed at 0 °C for 30 min in 2.5 % glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.4 (Buffer C). Pellets were washed several times and post fixed with 1 % OsO_4 in the same buffer for 1 h at 0 °C. Pellets were washed with several changes of Buffer C followed by several changes of distilled water. Material was stained en bloc with 0.5 % uranyl acetate for 1 h at 0 °C, followed by dehydration with a graded series of acetone. Embedding was in Epon 812 at 60 °C for 48 h. Sections were cut on a diamond knife (Dupont Instruments Company, Wilmington, Delaware) mounted on a Porter-Blum MT-2 ultramicrotome. Gray to silver sections were picked up with uncoated 400 mesh copper grids (Ted Pella Co., Tustin, Calif.). Sections were stained with 5 % (w/v) uranyl acetate at 60 °C for 15 min, followed by Reynolds lead citrate for 4 min at ambient temperature. Sections were observed with a Phillips EM 300 electron microscope operating at 60 kV, using a 50 μm objective aperature.

RESULTS

The scheme for the preparation of a plasma membrane enriched fraction is shown in Scheme 1. The important features of this method are perfusion of the liver, gentle homogenization in an isotonic medium at physiological ionic strength, and the fact that the low speed pellet obtained after homogenization is directly subjected to flotation on a discontinuous sucrose density gradient. A crude plasma membrane is isolated from the 35 %–39 % sucrose interface. Further fractionation is achieved by vigorous mechanical disruption of this material in the Polytron homogenizer, followed by sedimentation through a second sucrose density gradient. A light fraction occurs as a float at the sample buffer-32 % sucrose interface, while a heavy fraction is isolated from the 34 %–40 % sucrose interface. A pellet is also obtained, which has not been further analyzed.

The enzymatic profile of the various membrane fractions is shown in Table I. The average recovery of phosphodiesterase I is 32 % in the crude plasma membranes, which is equal to a relative specific activity of 24 for this marker over the homogenate. The extent of contamination by other cellular organelles was determined by assaying the appropriate marker enzymes. Somewhat less than 1 % of mitochondrial marker, succinate dehydrogenase, was found in crude membranes, at a relative specific activity of 0.50. The recovery of endocytoplasmic reticulum marker, NADPH-cytochrome *c* reductase, was 0.9 %, with a relative specific activity of 0.7. Contamination by lysosomes and Golgi was greater. The recovery of acid phosphatase (lysosomes) was 3.5 % with a relative specific activity of 2 over the homogenate, while 14 % of the galactosyltransferase activity was recovered in the crude membrane, which gives a relative specific activity of 9 for this Golgi marker enzyme.

The light fraction obtained from gradient 2, contained 22 % of the phosphodiesterase I activity, with an 80-fold purification over the homogenate. Less than 0.1 % of the succinate dehydrogenase was found in this fraction, with a relative specific activity of 0.1. About 0.14 % of the microsomal marker was recovered in the light fraction, with a relative specific activity of about 0.5. Acid phosphatase recovery in the light fraction was 1.4, with a relative specific activity of about 4. This fraction also

TABLE I

YIELD AND PURIFICATION OF PLASMA MEMBRANE FRACTIONS FROM RAT LIVER

Enzyme activities in the homogenate are given as μmol/min per mg protein, except that for galactosyltransferase, which is in nmol/min per mg protein. Results are given as % of the total activity in the original homogenate, with relative specific activity, specific activity of the fraction divided by the specific activity of the original homogenate, in brackets. Standard deviations were determined from the results of six experiments.

Fraction	Protein (mg/gm liver)	Phospho-diesterase I	Succinate dehydrogenase	NADPH-cytochrome *c* reductase	Acid phosphatase**	Galactosyl-transferase**
Homogenate	216±22	0.10±0.024	0.01±0.005	0.26±0.007	0.05	0.13
Crude Membranes	3.05 ±0.46	32±4 (24.8±3)	0.603±0.23 (0.5±0.3)	0.84±0.15 (0.67±0.3)	3.5 (2.2)	14.5 (8.5)
Ray method membranes†	1.73	13 (16)	1.2 (1.4)	0.9 (1.2)	1.6 (2)	3 (3.6)
41 000 × *g* supernatant	0.374±0.18	0.45 (2.7)*	0.004 (0.26)***	0.08 (0.45)***	0.3 (0.37)	0.5 (1.6)
Light	0.42±0.09	22 (80)*	0.026 (0.048)***	0.14 (0.51)***	1.4 (4.0)	4.3 (12.3)
Heavy	0.3±0.2	4.1 (28)*	0.071 (0.23)***	0.13 (1.66)***	0.06 (0.77)	0.49 (5.9)

* Averages from two experiments, each performed with pooled livers from 24 rats.
** Average from three experiments, each using two rats.
*** Values of two experiments, each using two rats.
† Values of a single experiment, two rats.

contained 5 % of the galactosyltransferase originally present in the homogenate, with a relative specific activity of 12.

The heavy fraction contained 5 % of the phosphodiesterase I with a relative purification of 28-fold. NADPH-cytochrome *c* reductase was found to about the same extent as in the light fraction, i.e. 0.13 %, but had a somewhat higher relative specific activity. This was also true for succinate dehydrogenase. Less than 0.1 % of the acid phosphatase was found in this fraction, along with about 0.5 % of the Golgi marker enzyme.

Of the total protein present in the homogenate about 1.5 % was recovered in the crude plasma membranes. Less than one-fifth of this protein was rendered soluble by the polytron treatment, and about another one-fifth was found in each of the light and heavy fractions. The remainder was presumably associated with the substantial pellet found at the bottom of the centrifuge tube, which was not analyzed. This 5-fold reduction in protein, coupled with the 75 % recovery of phosphodiesterase I, accounts for the approximately 4-fold increase in the specific activity of this enzyme in the light subfraction compared to the original crude plasma membrane.

Table I also includes results from one experiment where the plasma membrane fraction was prepared by a hypotonic method [18]. The yield of protein and phosphodiesterase I is comparable to that reported by others [19, 20], but less than one-half of that obtained by the present procedure, and with a somewhat smaller enrichment. The relative specific activity of succinic dehydrogenase and NADPH-cytochrome *c* reductase in the hypotonic membranes are also several-fold higher than in our crude plasma membranes; that of the lysosomal marker is about the same in the two preparations, while the relative specific activity of the Golgi element marker in the hypotonic preparation is less than one-half of that seen in our crude plasma membrane.

It is customary to calculate contamination by other subcellular organelles from the ratio of the relative specific activity for the appropriate marker enzymes in the plasma membrane and the purified organelle itself [21, 22]. Estimates of the latter differ considerably, but using a relative specific activity of 4 for succinic dehydrogenase in purified rat liver mitochondria [23], 6 for NADPH-cytochrome *c* reductase in endoplasmic reticulum (refs. 24–26 and Brown, A. E., Lok, M. P. and Elovson, J., unpublished), 70 for acid phosphatase in lysosomes [23, 27] and 100 for galactosyltransferase in the Golgi fractions (refs. 24, 28, 29 and Brown, A. E., Lok, M. P. and Elovson, J., unpublished) our crude plasma membranes could contain 7, 11, 5 and 9 %, respectively, of these contam-

TABLE II

CHEMICAL ANALYSIS OF VARIOUS MEMBRANE FRACTIONS

Cholesterol is given as μmol/mg protein; DNA and RNA values are from pooled material of 24 livers and are given as μg/mg protein. n.d., not determined.

Component	Fraction		
	Homogenate	Crude membranes	Light membranes
Cholesterol	0.03	0.36	1.07
DNA	n.d.	27.3	8.6
RNA	n.d.	35.3	22.9

inating organelles on a protein basis. As seen from Table I, about the same percentages would also be found in the light subfraction, while the hypotonic membranes could contain as much as 30 and 20 % mitochondrial and endoplasmic reticulum material, respectively. However, selective loss of marker enzymes in some fractions cannot be excluded, even if overall recoveries are good, and the "pure" organelle fractions themselves, such as microsomes, may show highly heterogeneous distribution of the marker enzymes among individual elements [30, 31]. Furthermore, there is always a possibility that some "foreign" marker enzymes may be genuine compo-

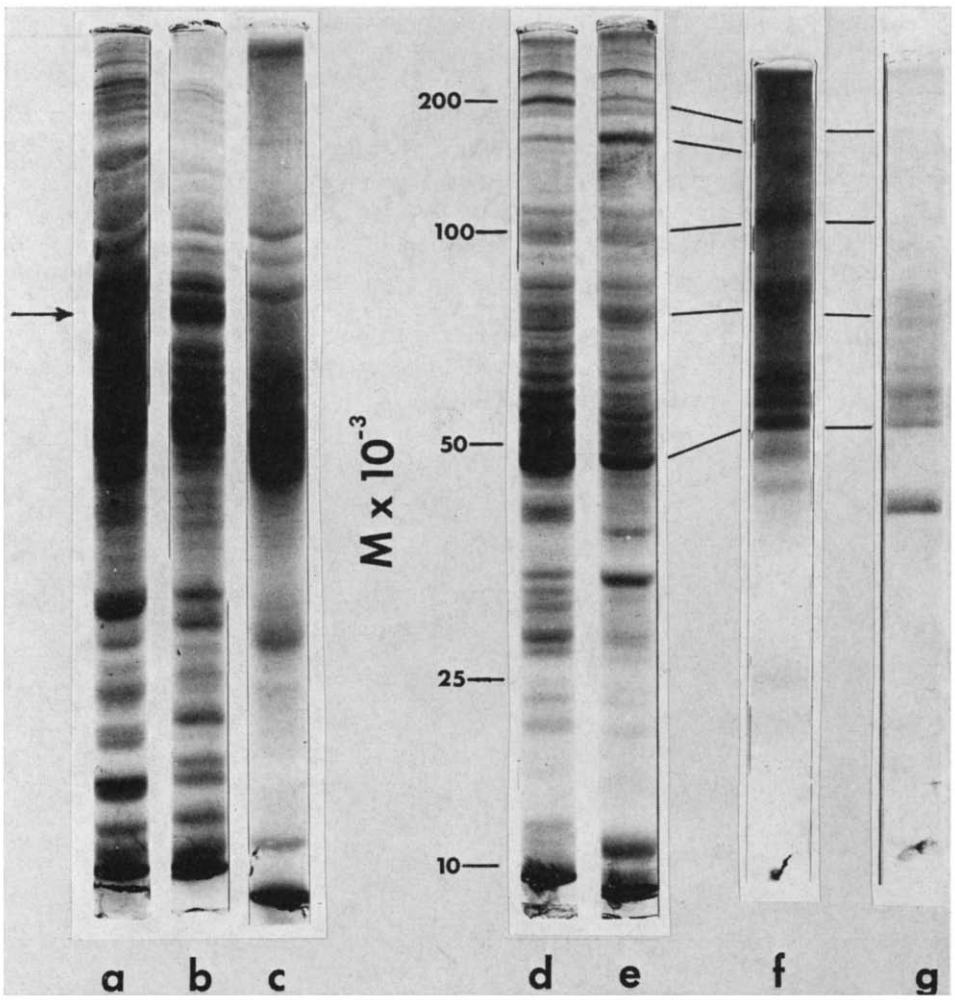
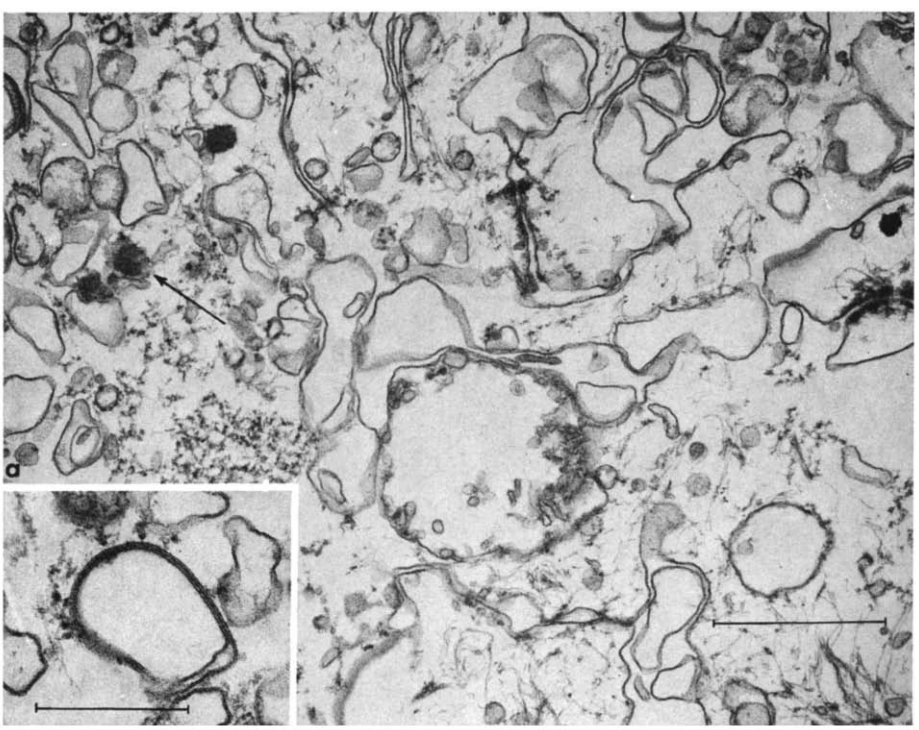


Fig. 1. Acrylamide gels of (a) Golgi fraction 1 (98 μg protein); (b) Golgi fraction 2 (77 μg protein); (c) Golgi fraction 3 (89 μg protein); (d) plasma membranes (75 μg protein) prepared by the hypotonic method of Ray [18]; (e) crude plasma membranes (75 μg protein); (f) light fraction (50 μg protein); (g) heavy fraction (68 μg protein). Variability of protein migration is due to membrane samples being run at different times. Identity of bands, as shown by lines, was established by running membrane samples simultaneously on acrylamide slab gels (not shown).

nents of the plasma membrane itself. Thus, although the galactosyltransferase is usually considered a marker for Golgi elements [28], this and other glycosyltransferases have also been claimed to be present in plasma membranes [32, 33], including that from rat liver [34]. Since, at this point, these questions remain unresolved, calculations such as the above can only be taken as very rough estimates of plasma membrane purity.

The cholesterol content of plasma membranes is characteristically much higher than in intracellular membranes. As seen in Table II, this value increases substantially from the crude to the light plasma membrane fractions, and the value for the latter is comparable to or higher than that reported for other preparations [19, 21, 22, 24, 40]. The amounts of nucleic acids in our plasma membrane fractions are also in the mid-range of those reported by others [21, 22, 24, 35].

The pattern obtained on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane fractions is shown in Fig. 1. Although some quantitative and qualitative differences are apparent, our crude plasma membrane and those obtained by the hypotonic Ray procedure [18] are quite similar, and show good agreement with the pattern previously reported by Neville and Glossmann [36]. About 28 protein bands are definable with a heaviest staining band at M_r 46 000 and a much less dense band at M_r 200 000, which comigrate exactly with rabbit muscle actin and myosin, respectively, when run in mixed samples (data not shown). The relative intensities of the bands also varies in the light and heavy subfraction but their patterns are basically similar to those seen for the crude membranes, except for an overall decrease in components of less than 35 000 molecular weight.



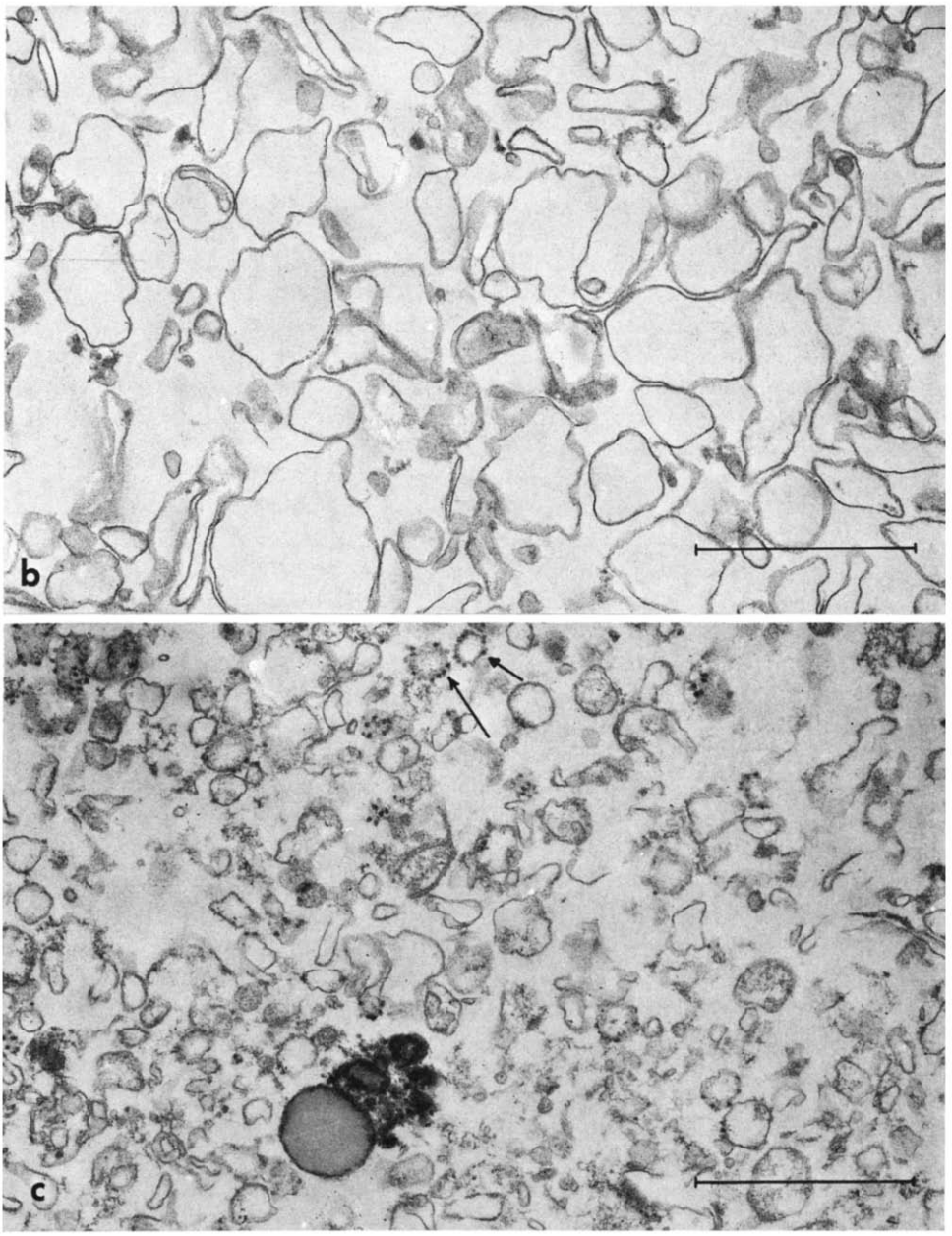


Fig. 2. (A) Thin section of crude plasma membranes. Nucleoids of microbodies (arrows) are present. Bar, 1 μm; magnification $\times$ 22 500. The insert shows a thin section of nexus found in the crude plasma membrane fraction. Bar, 0.5 μm; magnification $\times$ 40 500. (B) Thin section of the light membrane fraction showing the homogeneous nature of this membrane fraction. Bar, 1 μm; magnification $\times$ 29 250. (C) Thin section of the heavy membrane fraction. Arrows indicate vesicles of rough endoplasmic reticulum. Bar, 1 μm; magnification $\times$ 29 250.

The gel electrophoretic profile of the three Golgi subfractions obtained with the Ehrenreich procedure [5] is also shown in Fig. 1. Major bands in the two lighter fractions (Figs 1a and 1b) presumably represent cisternal serum proteins [45], one migrating at the position expected for rat serum albumin (arrow, Fig. 1a). However, although the three Golgi fractions clearly differ from the plasma membranes, the complexity of the banding patterns in both types of membranes (Fig. 1) make it difficult to determine which, if any, peptides might be present in both of them.

A thin section of the crude plasma membranes is shown in Fig. 2. In general, this fraction contained membrane sheets and vesicles of different size, some quite large; occasional gap junctions were also seen (insert, Fig. 2A). The preparation showed microbody nucleoids throughout, while the appearance of other contaminants such as mitochondria, Golgi- and lysosome-like bodies increased towards the bottom of the membrane pellets. The absence of nuclei in these sections was confirmed by light microscopy of the Trypan Blue-stained fraction. In contrast to the highly heterogeneous appearance of the crude plasma membrane, the light fraction was quite homogeneous, containing mostly large membrane vesicles, ranging in size from 0.3 to 1 μm (Fig. 2B). The heavy fraction (Fig. 2C) also contained mostly vesicles, but of smaller dimensions, 0.1–0.5 μm, many of which showed amorphous inclusions. Occasional vesicles carrying osmophilic granules presumably represent rough endoplasmic reticulum in accordance with the somewhat higher content of NADPH-cytochrome *c* reductase in this fraction. Neither the light nor the heavy subfraction showed recognizable junctional complexes; if not broken down beyond recognition by the Polytron treatment, they presumably sedimented into the pellet in the second gradient, which was not further analyzed.

Before adopting the final procedure, some other versions were also explored. Membranes were originally prepared on sucrose gradients which contained 1 mM $MgCl_2$ and 0.1 mM EDTA. This gave a somewhat higher yield of crude plasma membrane phosphodiesterase I, but much more protein was also recovered and the average purification was only about 16-fold, compared to 24-fold for the final method. Although a substantial proportion of this extra protein was solubilized by the Polytron treatment, both the specific activity and yield of phosphodiesterase I in the light fraction was consistently less than for the final version. Experiments were also done in which membranes from gradient I were fractionated by disruption either in the Polytron or by passage through a French pressure cell, followed by centrifugation on dextran or Ficoll gradients in the presence of mM Mg^{2+} [37]. The yield of phosphodiesterase I in the light fraction, which collected at the sample-16 % (w/v) dextran interface, was comparable to that obtained by the present method, but its specific activity was increased less than 1.5-fold over that in the crude plasma membrane. The French Press, but not the Polytron, was also found to render a major portion of both membrane protein and phosphodiesterase I activity non-sedimentable during centrifugation at 200 000 $\times g$ for 1 h.

DISCUSSION

The proliferation of methods of preparation of animal cell plasma membranes is a fair indication of the limitations in the established procedures. Several recent reviews of this general area are available [2–4] and we will confine our discussions

to the rat liver system investigated here, which is also the most thoroughly studied one. The most frequently used approach is the hypotonic treatment introduced by Neville [1], and its various modifications [18–20, 36], in which liver is gently disrupted in mM bicarbonate. After extensive dilution of the homogenate, a low-speed pellet is collected, which contains nuclei and larger fragments of plasma membrane; this is then washed several times in hypotonic medium, to remove smaller contaminants (mitochondria and microsomes). Plasma membrane fragments are then separated from nuclei and other remaining contaminants by flotation through a sucrose density gradient, typically collecting at a density of around 1.17. This material is generally agreed [2–4] to represent primarily a sample of the bile-front-interstitial hepatocyte surface, which has come through the mechanical shear as relative large membrane sheets, often with recognizable bile canaliculi [37], which are presumably stabilized by junctional complexes. Where determined, the recovery of protein (less than 1 mg/g liver) and 5'-AMPase (about 10 % of initial homogenate) are rather low, with an average about 20-fold enrichment of the latter, while the specific activities of markers for endoplasmic reticulum and mitochondria typically are somewhat less than in the homogenate [18–20, 38, 39, 49]. Although inclusion of 0.05 mM Ca^{2+} [18] in the medium was claimed to greatly improve yields in this procedure, the original data are somewhat hard to evaluate, and smaller or no effects were found by others [24, 38]. As a variation on the hypotonic method, Evans and coworkers [33, 39, 40] have routinely subjected the first low-speed pellet to rate-zonal centrifugation in a special rotor, to obtain a size range of plasma membrane which sediment between mitochondria and nuclei; their reported yield and purity are about the same as for the isopycnic separations. In his modified procedure, Neville also used a rate-zonal centrifugation step after the flotation to separate out the larger plasma membrane fragments [36]; however, as shown by others [20] this gives unacceptable losses without any further purification as estimated by assays for endoplasmic reticulum and modified markers. Rather similar yields and purities have also been obtained when essentially the same schedule of washings and centrifugations were applied to liver homogenates in isotonic sucrose [38, 41, 42].

A different approach has been to isolate small vesiculated plasma membrane elements from the microsomal fraction. One procedure was originally developed by Wallach [2, 3, 35], who proposed surface-change effects to account for the separation of plasma membrane and endoplasmic reticulum vesicles on Ficoll (or dextran) density gradients in the presence of divalent cations. Although these have been used to obtain plasma membrane fragments from liver also [24, 43], other methods have used simple sucrose density gradients with similar results [21, 22, 44]. Two very similar methods use both approaches. Touster et al. [21] subjected both the low-speed nuclear fraction and crude microsomes from an isotonic homogenate to isopycnic flotation on sucrose gradients, and recovered 10 and 20 %, respectively, of homogenate AMPase (as well as phosphodiesterase I) from the two fractions, as vesicles floating at density 1.16. House et al. [22] subjected the microsomal pellet to a preliminary separation on a Ficoll gradient, but otherwise followed the same protocol, and obtained very similar yields and purity. The plasma membrane marker enzymes in these preparations were about 20-fold enriched over homogenate, contamination with mitochondria was quite low, and the specific activity for endoplasmic reticular marker enzymes was about one-half [21] to one-fifth [22] of that in the homogenate.

The procedure described in this paper uses a different approach. In our hands, after gentle homogenization in isotonic KCl, three quarters of the original homogenate phosphodiesterase I activity are recovered in the initial low speed pellet, about one-half of which can be recovered as a "crude" plasma membrane fraction by direct flotation through a single isopycnic sucrose density gradient. So far, we have found no practical improvement on these simple steps. The extensive washings of the low speed pellet, which is used in most other procedures, certainly removes considerable amounts of mitochondria and microsomes, but in our hands the loss of small plasma membrane fragments is almost as large, compromising yields without commensurate improvement in purity. Homogenization in isotonic sucrose was also tried, but gave less consistent results. The yield, enrichment and purity in the crude plasma membrane fraction, as measured by protein, phosphodiesterase I and NADPH-cytochrome *c* reductase assays, is the same as that obtained in the more laborious Touster procedure [21], although contamination with mitochondria and lysosomes is somewhat higher. The morphology of this fraction is also more heterogeneous: large vesicles predominate, but considerable amounts of amorphous material are also seen with microbody nucleoids being easily recognizable contaminants. Vigorous mechanical disruption of the crude plasma membrane fraction followed by a second discontinuous sucrose gradient gave a vesicular light subfraction, with a combination of total yield (20 %) and enrichment (over 80-fold) for the plasma membrane marker enzyme which is notably higher than that obtained with any previously published method [21, 22, 39].

A less desirable feature of the present preparation is its high galactosyltransferase activity, which could suggest a considerable contamination with Golgi elements, although, as discussed above, some of this activity may be a genuine plasma membrane component. This is perhaps to be expected, considering the general similarity between our procedure and several methods for Golgi isolation [5, 45–47]. However, this is even more true for the Touster procedure, and several recent reports have shown enrichment of galactosyltransferase activity in such microsomal plasma membrane preparations to a level several-fold higher than that seen in our crude and light plasma membrane fractions [33, 48, 49]. It may be that this activity can be eliminated only by using methods which exclusively recover the large bile front fragment of the plasma membranes, with the attendant poor yields.

Evans previously reported the formation of a light subfraction, although in low yields, on vigorous rehomogenization of his bile front-enriched plasma membrane preparation [41], and has concluded from its pattern of marker enzymes and hormone receptors that it derives from the bile canalicular microvilli [50, 51], while the microsomal light fraction obtained in the Touster procedure would derive primarily from the sinusoidal microvilli [33]. Since in our method most of the homogenate phosphodiesterase I activity sediments at low speed, our crude membranes presumably contain larger fragments from both the bile canalicular and sinusoidal surfaces, suggesting that our light fraction contains a mixture of smaller vesicular elements from both these sides of the hepatocyte. A definitive resolution of these questions will have to await the unambiguous ultrastructural localization of plasma membrane markers in the cell, either by virtue of their enzymatic activities, or by immune-staining techniques [52]. As a first step towards this goal we have used our light fraction as a starting material in the purification of two plasma membrane proteins, one of which is the phosphodiesterase I itself (Elovson, J., unpublished).

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REFERENCES

1 Neville, D. M. (1960) J. Biophys. Biochem. Cytol. 8, 413
2 Steck, T. L. and Wallach, D. F. H. (1970) in Methods in Cancer Research (Busch, H., ed.), pp. 93–155, Academic Press, New York
3 Wallach, D. F. H. and Lin, P. S. (1973) Biochim. Biophys. Acta 300, 211
4 De Pierre, J. W. and Karnovsky, M. (1973) J. Cell Biol. 56, 275
5 Ehrenreich, J. H. ,Bergeron, J. J. M., Siekevitz, P. and Palade, G. E. (1973) J. Cell Biol. 59, 45
6 Spiro, R. G. (1964) J. Biol. Chem. 239, 567
7 Ostrowski, W. and Tsugita, A. (1961) Arch. Biochem. Biophys. 94, 68
8 Pennington, R. J. (1961) Biochem. J. 80, 649
9 Neville, D. M. (1971) J. Biol. Chem. 246, 6328
10 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606
11 Brown, A. E. and Elovson, J. E. (1974) Biochemistry 13, 3476
12 Rudel, L. L. and Morris, M. D. (1973) J. Lipid Res. 14, 364
13 Lowry, O. H., Rosebrough, N., Farr, J. and Randall, R. J. (1951) J. Biol. Chem. 193, 265
14 Fleck, A. and Munro, H. N. (1962) Biochim. Biophys. Acta 55, 571
15 Abraham, G. N., Scaletta, C. and Vaughan, J. H. (1972) Anal. Biochem. 49, 547
16 Giles, K. W. and Meyers, A. (1965) Nature 206, 93
17 Schneider, W. C. (1957) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.), Vol. 3, pp. 681–684, Academic Press, New York
18 Ray, T. K. (1970) Biochim. Biophys. Acta 196, 1
19 Emmelot, P., Bos, C. J., Benedetti, E. L. and Rümke, P. H. (1964) Biochim. Biophys. Acta 90, 126
20 Lauter, C. J., Solyom, A. and Trams, E. G. (1972) Biochim. Biophys. Acta 226, 511
21 Touster, O., Aronson, Jr., N. N., Dulaney, J. T. and Henrickson, H. (1970) J. Cell Biol. 47, 604
22 House, P. D. R., Poulis, P. and Weidemann, J. (1972) Eur. J. Biochem. 24, 429
23 Fleischman, R., Mattenheimer, H., Holmes, A. W. and Remmer, H. (1975) Biochem. Biophys. Res. Commun. 62, 289
24 Fleischer, S. and Kervina, M. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. 31A, pp. 34–35, Academic Press, New York
25 Tappel, A. L., Sawant, P. L. and Hibko, S. (1967) in Lysosomes (Reuck, A. V. S. and Cameron, M. P., eds.), p. 82, J. A. Churchill, London
26 Fleischer, B. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. 32A, p. 185, Academic Press, New York
27 Bergeron, J. J. M., Ehrenreich, J. H., Siekevitz, P. and Palade, G. E. (1973) J. Cell Biol. 59, 73
28 Dallner, G., Bergstrand, A. and Nilsson, R. (1968) J. Cell Biol. 38, 257
29 Widnell, C. C. (1972) J. Cell Biol. 52, 542
30 Roseman, S. (1970) Chem. Phys. Lipids 5, 270
31 Patt, L. M. and Grimes, W. J. (1974) J. Biol. Chem. 249, 4157
32 Pricer, Jr., W. E. and Ashwell, G. (1971) J. Biol. Chem. 246, 4825
33 Wisher, M. H. and Evans, W. H. (1975) Biochem. J. 146, 375
34 Neville, D. M. and Glossman, H. (1971) J. Biol. Chem. 246, 6335
35 Kamat, V. B. and Wallach, D. F. H. (1965) Science 148, 1343
36 Neville, Jr., D. M. (1968) Biochim. Biophys. Acta 154, 540
37 Song, C. S., Rubin, W., Rifkind, A. B. and Kappas, A. (1969) J. Cell Biol. 51, 124
38 Hawkins, C. F. and Jacquez, J. A. (1972) Anal. Biochem. 49, 290
39 Evans, W. H. (1969) FEBS Lett. 3, 237

40 Evans, W. H. (1970) Biochem. J. 116, 833
41 Berman, H. M., Gram, W. and Spirtes, M. A. (1969) Biochim. Biophys. Acta 183, 10
42 Prospero, T. D., Burge, M. L. E., Norris, K. A., Hinton, R. H. and Reid, E. (1973) Biochem. J. 132, 449
43 Graham, J. M., Higgins, J. A. and Green, C. (1968) Biochim. Biophys. Acta 150, 303
44 Stein, Y. C., Widnell, C. and Stein, O. (1968) J. Cell Biol. 39, 185
45 Fleischer, B. and Fleischer, S. (1970) Biochim. Biophys. Acta 219, 301
46 Morre, D. J., Cheetham, R. D., Nyquist, S. E. and Ovtracht, L. (1972) Prep. Biochem. 2, 61
47 Leelavathi, D. E., Estes, L. W., Feingold, D. S. and Lombardi, B. (1970) Biochim. Biophys. Acta 211, 124
48 Dewald, B. and Touster, O. (1973) J. Biol. Chem. 248, 7223
49 Lishmore, G. S., Tulsiani, D. R. P., Bhavanandan, V. P. and Carbelli, R. (1975) J. Biol. Chem. 250, 2655
50 Evans, W. H., Bergeron, J. J. M. and Geschwind, I. I. (1973) FEBS Lett. 34, 259
51 Wisher, M. H. and Evans, W. H. (1974) Biochem. Soc. Trans. 2, 407
52 Painter, R. G., Tokuyasu, K. T. and Singer, S. J. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1649