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A RAPID HIGH YIELD METHOD FOR THE PREPARATION OF RAT LIVER CELL PLASMA MEMBRANES

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SUMMARY

This report describes a simple method for the simultaneous isolation of a number of samples of rat liver plasma membranes, with high yield, purity and reproducibility.

Yield and purity are discussed in terms of criteria used in other published procedures.

INTRODUCTION

Many methods for the isolation of liver cell plasma membranes are now available[1–9], but in our experience they all suffer from one or more of the following disadvantages: (a) low yield, (b) long, involved preparative procedures, (c) unsuitability for the simultaneous isolation of a number of plasma membrane samples, (d) high level of contamination by organelles, (e) lack of membrane fragments representative of the total hepatocyte surface, and (f) unsuitability for the isolation of comparable samples of plasma membranes from the livers of rats treated *in vivo* with drugs or toxins, or following surgical manipulation. A method which overcomes these disadvantages is essential for studies of the plasma membrane in cell pathology.

A method is described here which allows the preparation of three very pure samples of plasma membranes, at high yield, from single rat livers or large hepatomas by a single worker in 5–6 h. This method was developed following failure to isolate plasma membranes from autolysing rat liver by other published methods. This method allows the simultaneous isolation of comparable samples of plasma membranes from the livers of both treated and untreated animals (as judged by yield and purity studies), and has been employed in studies following treatment with a wide range of hepatocellular toxins.

Although this present method was developed to satisfy the author's own requirements, it may be applicable to the isolation of plasma membranes from a wide range of tissues, and may be of interest especially where a reduction of preparation time is desirable.

METHODS

Animals

Male Sprague–Dawley rats (200–250 g body wt) (Animal House, Department of Pathology, University of Melbourne) were fed a wholly pelletted diet containing

18% protein and less than 1% calcium, and water *ad libitum*. Animals were fasted 18 h before being killed by stunning and exsanguination.

Preparation of plasma membranes

A liver (about 5 g) was placed in an ice-cold buffer solution of 1 mM $Na_2B_4O_7$, 0.5 mM $CaCl_2$, pH 7.5, cut into small pieces and washed free of blood. The tissue was dispersed in a loose fitting Dounce homogenizer (radial clearance 0.006 inch Blaessig Glass Co., Rochester, N.Y.) with five strokes (10 ml of medium/g liver). This partial homogenate was centrifuged at $150 \times g$ for 10 min in 100 ml tubes in a refrigerated centrifuge (2 °C). The supernatant was decanted into a receiving vessel. The nuclear pellet was resuspended twice in the same tube in 50 ml of medium and centrifuged as before. Resuspension was accomplished using a teflon pestle (radial clearance 0.02–0.04 inch inside selected 100 ml tubes) rotating at 1000 rev./min. The three supernatants were pooled and the final nuclear pellet was discarded. The pooled supernatant was centrifuged at $2000 \times g$ for 10 min and the resultant supernatant was removed by aspiration. The pellet was washed once in 100 ml and then in 15 ml of buffer (in a graduated conical centrifuge tube). The pellet (about 4 ml) was resuspended to 15 ml with 70% sucrose (w/w) and underlayed in a discontinuous sucrose gradient (2 ml 54%, 2 ml 49%, 5 ml 45%, 5 ml 41% and 3 ml 37% sucrose, w/w, respectively) in a Beckman 1 inch × 3 inch cellulose nitrate tube. The prepared gradient was centrifuged in an S.W. 25.1 rotor for 100 min at 25000 rev./min at a rotor temperature of −5 °C in a Beckman L2 65B ultracentrifuge. A solidly packed band at the interphase 37–41% sucrose ($d = 1.16$–1.18) was harvested with a Pasteur pipette and was twice washed with 7 mM Tris buffer, pH 7.5, and finally resuspended in the same buffer to give a protein concentration between 2 and 2.5 mg/ml.

Enzyme estimations

Assays of 5′-nucleotidase (EC 3.1.3.5) were made according to the method of Emmelot and Bos[10] with AMP (Sigma) and phenyldisodium orthophosphate (BDH) as substrates for 5′-nucleotidase and interfering non-specific phosphatases, respectively. Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed by the method of de Duve *et al.*[11] using glucose 6-phosphate (Sigma) as substrate. Acid phosphatase (EC 3.1.3.1) was assayed by the method of Gianetto and de Duve[12] with β-glycerophosphate (Sigma) as substrate. The succinate cytochrome *c* reductase system was assayed by the method of de Duve *et al.*[11] using reagents obtained from Sigma and BDH. Mg^{2+}-ATPase (EC 3.6.1.3) and $(Na^+ + K^+)$-activated ATPase were estimated by the method of Emmelot and Bos[10] with Tris–ATP (Sigma) as substrate. Monoamine oxidase was determined spectrophotometrically by the method of Schnaitman *et al.*[13]. Alkaline phosphatase was determined by the method of Ray[9].

Chemical determinations

Liberated inorganic phosphorus was estimated by a modification of the method of Fiske and SubbaRow[14] as described by Bartlett[15], applied to an autoanalyser essentially of the Technicon design and adapted to a Hitachi 101 spectrophotometer and QPD 53 recorder. Protein was determined by a modification of the method of Lowry *et al.*[16] using crystalline bovine serum albumin as standard. DNA and RNA were determined by the method of Schneider[17].

Lipids were extracted from purified plasma membranes by the method of Folch *et al.*[18]. Total lipids were estimated by the method of Johnson[19], cholesterol by the method of Jurand and Albert-Recht[20], and total phospholipids by the organic phosphorus method of Bartlett[15]. Phospholipid classes were separated by two-way thin-layer chromatography over silica gel (Mercke GF254) slurried with 5% $(NH_4)_2SO_4$ and developed with the following solvent mixtures: chloroform–methanol–water (65:25:4, by vol.) and butanol–acetic acid–water (60:20:20, by vol.). Phospholipid spots were taken from the plates and the phosphorus was estimated by the method of Bartlett[15].

Electron microscopy

Plasma membrane pellets were fixed in glutaraldehyde, post fixed in OsO_4 and embedded in araldite 812. Thin sections stained with U and Pb were examined with a Siemen's Elmiskop I electron microscope.

RESULTS

85–90% of the total homogenate 5′-nucleotidase activity was present in the pooled supernatant obtained following washing of the nuclear pellet and the crude plasma membrane fraction applied to the sucrose gradient contained 35–40% of the total 5′-nucleotidase activity. The purified plasma membrane fraction harvested, was localised as a tightly packed layer at the $d = 1.16$–1.18 interface. Smaller slower moving fragments rich in 5′-nucleotidase activity were located at the $d = 1.18$–1.20

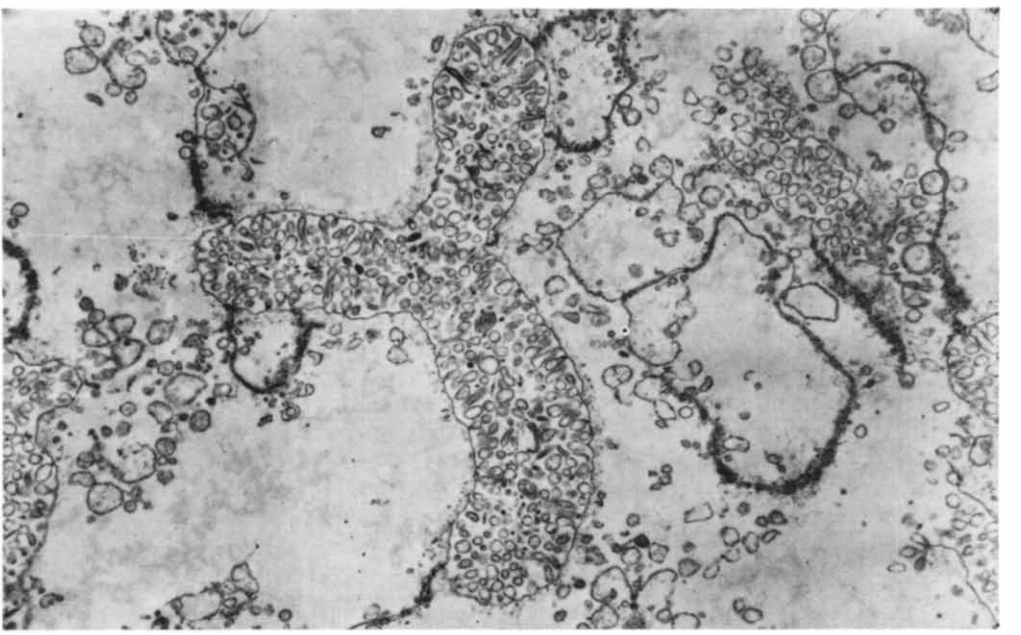

Fig. 1. An electron micrograph of a liver plasma membrane preparation from an untreated rat. In the centre of the field there is a large profile of a bile canaliculus containing many microvilli in section. The bile canaliculus is attached to pieces of double membrane at five different places. Other pieces of membrane and microvilli in section are scattered around the profile. Magnification: × 6000.

TABLE I

ACTIVITIES AND YIELDS OF MARKER ENZYMES

Specific activities of the phosphatases are expressed as μmoles inorganic phosphorus/h per mg membrane protein $\pm$S.D. Succinate cytochrome *c* reductase activity is expressed as μmoles reduced per min per mg membrane protein $\pm$S.D. Percent recovery equals total preparation activity as a percentage of the total homogenate activity. Relative specific activity equals the ratio of the preparation specific activity to homogenate specific activity. Results are given as the mean of four determinations.

Preparation	*mg membrane protein/g wet wt liver*	*5'-Nucleotidase*			*Glucose-6-phosphatase*		*Acid phosphatase*		*Succinate cytochrome c reductase*	
		% Recovery	*Specific activity*	*Relative specific activity*	*% Recovery*	*Specific activity*	*% Recovery*	*Specific activity*	*% Recovery*	*Specific activity*
1	1.5	16.9	85.2 $\pm$ 2.8	25.3	0.8	1.15 $\pm$ 0.07	0.2	0.37 $\pm$ 0.02	0.09	0.025 $\pm$ 0.002
2	1.5	16.5	88.0 $\pm$ 7.8	25.2	0.9	1.09 $\pm$ 0.06	0.3	0.36 $\pm$ 0.02	0.08	0.027 $\pm$ 0.001
3	1.5	18.5	86.6 $\pm$ 4.8	25.6	0.9	1.08 $\pm$ 0.09	0.3	0.34 $\pm$ 0.03	0.04	0.012 $\pm$ 0.002

interface, but these were not collected as they were too difficult to harvest without contamination by mitochondrial fragments. Data related to yield, purity and reproducibility of this method is contained in Table I. Fig. 1 is an electronmicrograph of Prepn 2 (see Table I).

Table II contains data relevant to plasma membrane samples prepared by this method. This data represents accumulated control data from samples other than those referred to in Table I, and were subject to the same criteria of purity as those referred to in Table I.

TABLE II

COMPOSITION OF PLASMA MEMBRANES

Values are given as ranges and means ± S.D. Numbers in parentheses represent number of pieces of data collected. Specific activity is expressed as μmoles P_i released per h per mg protein. Yield represents total activity recovered as a percentage of total homogenate activity.

5′-Nucleotidase (specific)	76–94	(10)
Mg^{2+}-ATPase (specific)	110–140	(10)
$(Na^{+}+K^{+})$-ATPase (specific)	4–7	(10)
Monoamine oxidase	undetected	(4)
5′-Nucleotidase (% yield)	16–24	(8)
Alkaline *p*-nitrophenol phosphatase	20–27	(4)
mg total lipid/mg membrane protein	0.80 ± 0.13	(5)
Cholesterol (% total lipid)	23.5 ± 2.2	(5)
Phospholipids (% total lipid)	56.7 ± 5.7	(5)
Individual phospholipid phosphorus (% total lipid phosphorus)		
Lysophosphatidyl choline	2.4 ± 1.8	(4)
Sphingomyelin	19.3 ± 2.8	(4)
Phosphatidylcholine	31.0 ± 4.4	(4)
Phosphatidylinositol	8.8 ± 2.9	(4)
Phosphatidylethanolamine	19.7 ± 3.1	(4)
Phosphatidylserine	9.0 ± 5.1	(4)
Phosphatidylglycerol	4.5 ± 1.2	(4)
Phosphatidic acid and cardiolipin	4.3 ± 2.9	(4)
μg DNA/mg membrane protein	undetected	(6)
μg RNA/mg membrane protein	15–18	(6)
Cholesterol/phospholipid (molar)	0.83	

DISCUSSION

The hepatocyte plasma membrane is not a uniform structure, it includes bile canaliculi, desmosomes and tight junctions as components of intercellular membranes, and the sinusoidal surface with its numerous microvilli. Subfractions of different regions show differences in density[21]. Vigorous homogenization of liver, as is necessary when using isotonic buffers, may result in excessive fragmentation of the surface membrane and thus the isolation of a sample enriched in one particular type of fragment. Such differences may have contributed to the variation in values published for the density of rat liver plasma membranes.

In the present method a hypotonic buffer was used in the interest of rupturing

the cell membrane without excessive fragmentation. Dispersion of liver tissue in a loose fitting Dounce homogenizer or in the case of hepatoma, being forced through a tissue press, followed by osmotic shock resulted in large membrane fragments which moved rapidly on sucrose gradients. The appearance of plasma membrane fragments (Fig. 1) suggests that plasma membranes isolated by this method have not been subjected to excessive fragmentation. Their appearance is in many respects identical to that seen in whole liver. In this type of procedure the use of a buffer containing Ca^{2+} is well documented[6,9,22]. Of the buffers tested, the one containing borate gave the best yield. At no stage with this buffer did nuclei clump to form a gelatinized mass as was the case when $NaHCO_3$ buffer was used. It is possible that this was the reason for the higher yield when borate buffer was employed (*i.e.* the ease with which membrane fragments were removed from the nuclear pellet). By phase contrast microscopy nuclei appeared as discrete bodies. The effect of borate buffer in this respect may be due to borate combining with carbohydrate residues on membrane surfaces, increasing the net surface charge and thus favouring repulsion as opposed to attraction[23,24].

In this study an attempt was made to relate to a single preparation the varied criteria for plasma membrane yield and purity used by many workers. Such parameters include percentage yield of a number of enzymes, yield expressed as mg membrane protein/g wet wt liver, assessment of content of marker enzymes and chemical constituents of possible contaminating organelles, as well as factors pertaining directly to the plasma membrane itself *e.g.* relative specific activity of marker enzymes and cholesterol:phospholipid molar ratios.

It is the opinion of the authors that 5′-nucleotidase may well be the most useful single marker enzyme for the plasma membrane. Although there has been considerable controversy over the exact localization of this enzyme as interpreted by fractionation studies[26,27], it has been accepted as a specific plasma membrane marker from histochemical studies, where it is considered to be specifically localised in the bile canalicular membrane and the sinusoidal surface of the hepatocyte[28,29,30]. Too little is known about the fragmentation and vesiculation of microvilli of the plasma membrane and their possible movement with the microsomal fraction to justify a firm conclusion on fractionation studies alone. Because of the more widespread distribution of 5′-nucleotidase on the hepatocyte surface it is probably a more useful marker enzyme than alkaline phosphatase which is localised only on bile canalicular membranes[31,32]. The percentage yield of alkaline phosphatase can only be interpreted as percentage yield of bile canaliculi and not the total cell surface.

This present method compares well with the best of other published procedures for yield *i.e.* 16–18% recovery of total homogenate 5′-nucleotidase activity and 1.5 mg membrane protein/g wet wt liver (Table I), and purity *i.e.* 5′-nucleotidase relative specific activity 25–26, low activity of marker enzymes of possible contaminating organelles, and a cholesterol/phospholipid molar ratio of 0.83 (Tables I and II). Rat liver plasma membranes prepared by this method have a lipid content comparable to other published data[25] (Table II). This present method also has the following advantages:

(1) It is applicable to the simultaneous preparation of a number of plasma membrane samples. This feature is essential for studies following *in vivo* treatment with any compound. With the use of more modern high speed rotors six separate

samples could be prepared by this method at one time, in yields high enough for extensive studies.

(2) It is a useful general method for the preparation of plasma membranes from both normal and abnormal livers, resulting in comparable samples when applied to both types of isolation.

(3) These preparations can be made by a single worker in 5–6 h (including handling and centrifugation time). Short preparation time is essential for studies of labile components of plasma membranes *e.g.* saturation of fatty acyl groups of phospholipids.

(4) Very little handling and glassware is required to carry out this simple procedure.

(5) By this method very large fragments of plasma membranes can be prepared, some of which may represent the total hepatocyte surface. Since 5′-nucleotidase activity is associated with both the bile canalicular and sinusoidal surfaces[28–30] and since alkaline phosphatase activity is associated with bile canaliculi only[31,32], a preparation which contains equal yields of both enzyme activities would be expected to contain fragments of plasma membranes which are representative of the total hepatocyte surface. By this method preparations can be made which contain 16–24% and 20–27% yields of 5′-nucleotidase and alkaline phosphatase activities, respectively (Table II), which would indicate an approximately representative sample (*cf.* the method of Ray[9], 38% alkaline phosphatase and about 12% 5′-nucleotidase calculated from protein content).

This method has been used by the authors in a study of the involvement of plasma membrane lipid associated and lipid dependent enzymes in a number of pathological states. It has also been used in the preparation of liver cell plasma membranes of sheep and several strains of mice.

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REFERENCES

1 Neville Jr, D. M. (1960) *J. Biophys. Biochem. Cytol.* 8, 413–422
2 Emmelot, P., Bos, C. J., Benedetti, E. L. and Rümke, Ph. (1964) *Biochim. Biophys. Acta* 90, 126–145
3 Herzenberg, L. A. and Herzenberg, A. L. (1962) *Proc. Natl. Acad. Sci. U.S.* 47, 762–767
4 Takeuchi, M. and Terayama, H. (1965) *Exp. Cell Res.* 40, 32–44
5 Coleman, R., Michell, R. H., Finean, J. B. and Hawthorne, J. N. (1967) *Biochim. Biophys. Acta* 135, 573–579
6 Berman, H. M., Gram, W. and Spirtes, M. A. (1969) *Biochim. Biophys. Acta* 183, 10–18
7 Touster, O., Aronson N. N. Jr, Dulaney, J. T. and Hendrickson, H. (1970) *J. Cell Biol.* 47, 604–618
8 Weaver, R. A. and Boyle, W. (1969) *Biochim. Biophys. Acta* 173, 377–388
9 Ray, T. K. (1970) *Biochim. Biophys. Acta* 196, 1–9
10 Emmelot, P. and Bos, C. J. (1966) *Biochim. Biophys. Acta* 120, 369–382
11 De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) *Biochem. J.* 60, 604–617

12 Gianetto, R. and De Duve, C. (1955) *Biochem. J.* 59, 433–438
13 Schnaitman, C., Erwin, V. G. and Greenawalt, J. W. (1967) *J. Cell Biol.* 32, 719–735
14 Fiske, C. H. and SubbaRow, Y. (1925) *J. Biol. Chem.* 66, 375–400
15 Bartlett, G. R. (1959) *J. Biol. Chem.* 234, 466–468
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
17 Schneider, W. C. (1957) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 3, pp. 680–684, Academic Press, New York
18 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.*, 226, 497–509
19 Johnson, M. J. (1949) *J. Biol. Chem.* 181, 707–711
20 Jurand, J. and Albert-Recht, F. (1962) *Clin. Chim. Acta* 7, 522–528
21 Evans, W. H. (1970) *Biochem. J.* 116, 833–842
22 Emmelot, P. and Bos, C. J. (1969) *Int. J. Cancer* 4, 705–722
23 Zittle, C. A. (1951) *Adv. Enzymol.* 12, 493–527
24 McCollester, D. L. (1970) *Cancer Res.* 30, 2832–2840
25 Ray, T. K., Skipski, V. D. Barclay M.. Essner, E. and Archibald, F. M. (1969) *J. Biol. Chem.* 244, 5528–5536
26 Song, C. S. and Bodansky, O. (1967) *J. Biol. Chem.* 242, 694–699
27 Song, C. S., Kappas, A. and Bodansky, O. (1969) *Ann. N.Y. Acad. Sci.* 166, 565–573
28 Wachstein, M. and Meisel, E. (1957) *Amer. J. Clin. Path.* 27, 13–23
29 Essner, E., Novikoff, A. B. and Masek, B. (1958) *J. Biophys. Biochem. Cytol.* 4, 711–716
30 Goldfischer, S., Essner, E. and Novikoff, A. B. (1964) *J. Histochem. Cytochem.* 12, 72–95
31 Wachstein, M. and Zak, F. G. (1946) *Proc. Soc. Exp. Biol. (N.Y.)* 62, 73–76
32 Wachstein, M. and Zak, F. G. (1949) *Proc. Soc. Exp. Biol. (N.Y.)* 72, 234–236