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A SIMPLE METHOD FOR THE ISOLATION OF RAT LIVER CELL PLASMA MEMBRANES IN ISOTONIC SUCROSE

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SUMMARY

A method for the isolation of plasma membranes from rat liver parenchymal cells is described. It involves homogenization of parenchymal cell-enriched liver pulp in isoosmotic sucrose in a loose-fitting Dounce homogenizer. The nuclear fraction containing membranes is recovered by centrifugation. This is followed by elution of plasma and other membranous elements with either isotonic saline, isotonic saline containing 0.5 mM $CaCl_2$ or isotonic saline containing 2 mM EDTA and their final purification by discontinuous sucrose density gradient centrifugation. The plasma membranes are recovered at the 1.16–1.18 sucrose density interface: with isotonic saline, 0.37 mg plasma membrane protein per g wet weight rat liver is recovered. A comparison with other methods indicates that the preparation described is the simplest one available presently for cell homogenates made in isoosmotic sucrose.

INTRODUCTION

Isolation of plasma membranes from liver cells has been described by a number of workers[1–6]. The procedure described by NEVILLE[1] has been modified by EMMELOT *et al.*[2], and they have extensively used it for the study of enzymes in the plasma membranes of rat liver cells. However, this procedure involves homogenization of liver in ice-cold water buffered with 1 mM $NaHCO_3$, and for that account it has been criticized by TAKEUCHI AND TERAYAMA[4] and by BERMAN *et al.*[6]. In our experience, the chief difficulties with the method according to NEVILLE[1] lie in large volumes to be used, gelatinization of particulate matter during homogenization or when the homogenate is allowed to stand for 0.5–1 h on ice so that separation between sediment and supernatant at low speed is not well defined, and that the liver homogenate used for the preparation of plasma membranes can hardly be utilized further for the isolation of other cellular components. In these respects, procedures utilizing isotonic solutions seem to be more desirable and are presumably less harmful to the other cellular components such as mitochondria and nuclei. STEIN *et al.*[7] and TAKEUCHI AND TERAYAMA[4] used isoosmotic sucrose containing $MgCl_2$ and $CaCl_2$, respectively, whereas BERMAN *et al.*[6] employed buffered isoosmotic sucrose containing $CaCl_2$ for the isolation of rat liver plasma membranes. From the washed crude nuclear fraction, TAKEUCHI AND TERAYAMA[4] isolated plasma membranes by two successive sucrose

density gradient centrifugations. Similarly BERMAN *et al.*[6] collected a fraction sedimenting between 150 and 2000 × *g*, and purified plasma membranes from it by two sucrose density gradient centrifugations. We have noted that this (150–2000 × *g*) fraction obtained in method according to BERMAN *et al.*[6] is heavily contaminated with various particulate fractions such that the first sucrose density gradient centrifugation does not give a clear separation of the plasma membrane band. Moreover, it has been our experience that the presence of a divalent salt such as $CaCl_2$ in the homogenate leads to the agglutination of various cellular components and their sedimentation with the nuclear fraction. In order to minimize the difficulties observed with the above mentioned methods, we have resorted to the initial use of isoosmotic sucrose for homogenization and washing of the nuclear fraction containing membranes, followed by recovery of the plasma membrane fraction by centrifugation at low speed after suspension of the nuclear fraction containing membranes in isotonic NaCl. While this paper was in preparation, TOUSTER *et al.*[8] reported on the preparation of rat liver plasma membranes in isotonic sucrose.

MATERIALS AND METHODS

Preparation of liver cell membranes

Sprague–Dawley male rats of an average weight of 175 g were sacrificed by decapitation and their livers perfused *in situ* with ice-cold 0.9% NaCl. The livers were excised, chilled on ice for 2 min, blotted on filter paper, and were then minced in a cold plexiglass squeezer as described by DE LAMIRANDE[9]. This procedure results in an enrichment of parenchymal cells. The pulp thus obtained (10 g from two rat livers) was homogenized in ice-cold isoosmotic sucrose in a loose-fitting Dounce homogenizer (Kontes Glass Co., Vineland, N.J.) kept under ice, giving 26 up and down strokes. All subsequent operations were carried out between 0–5°. The total volume of the homogenate was 75 ml. The homogenate was centrifuged in plastic tubes in an International refrigerated centrifuge (Head No. C2420) at 4200 rev./min (2000 × *g*) for 15 min. The supernatant containing submicroscopic components was removed and the sediment was suspended in 75 ml of ice cold isoosmotic sucrose and recentrifuged at the above speed for 15 min. The pellet obtained was then suspended in isotonic saline, rehomogenized in the Dounce homogenizer giving only 4 up and down strokes. The suspension was centrifuged at 4200 rev./min as above for 10 min and the supernatant was discarded. The well packed pellet was suspended twice in 75 ml of either of the three mediums: isotonic saline, containing 0.5 mM $CaCl_2$ or isotonic saline containing 2 mM EDTA. The suspension was centrifuged at 1800 rev./min (350 × *g*) for 10 min. The sediment containing nuclei, mitochondria and cell fragments was discarded and the supernatants were combined and centrifuged at 25000 rev./min in a Spinco Model L ultracentrifuge (Rotor 30) for 15 min. The sediment was freed of solution by inverting the centrifuge tubes and wiping the sides with absorbant paper. The pellet was suspended in 5–6 ml of a 59.9% sucrose solution (density 1.22) and gently homogenized in the loose-fitting Dounce homogenizer. The suspension was transferred to plastic tubes for discontinuous sucrose density gradient centrifugation. The density of the sucrose solutions (wt./vol.) was determined at 20–22° using a refractometer (Officine Galileo). Over 5 ml of 59.9% sucrose suspension of membranes were subsequently layered, 5 ml of 54.1% sucrose solution (density 1.20), 9 ml of

48.45 % sucrose solution (density 1.18) and 8 ml of 42.9 % sucrose solution (density 1.16). The tubes were centrifuged in the Spinco Rotor 25.1 for 75 min at 90000 × *g*. The density gradient centrifugation gave three membrane fractions (bands), one at each of the interfaces and a small sediment at the bottom of the tube. A small amount of material floating on top of the gradient was removed and the three bands were collected with a syringe. They were pelleted by centrifugation at high speed (25000 rev./20 min) after dilution with distilled water. The pellets were resuspended in water by gentle homogenization and used as such.

Every step of the fractionation was monitored with phase contrast and electron microscopes.

Preparation of liver cell organelles

The supernatant obtained after centrifugation of the homogenate at 4200 rev./min was submitted to differential centrifugation according to the method of DE DUVE *et al.*[10]. The mitochondrial, lysosomal and microsomal pellets obtained after fractionation were resuspended in distilled water and used as source of enzymes.

Electron microscopy

The pellets obtained during the fractionation were fixed overnight in 2 % cacodylate-buffered glutaraldehyde (pH 7.4). They were then washed in the cacodylate buffer, and small slices were postfixed for 2 h in 1.3 % collidine-buffered OsO_4 (pH 7.2). After dehydration in alcohol, the slices were embedded flat in an Epon mixture. The complete cross sections of oriented slices, parallel to their top to bottom axis, were obtained with a Porter–Blum ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a JEM-7A electron microscope.

Enzymatic and chemical assays

All absorbance measurements were determined with a Beckman DU spectrophotometer. Cytochrome oxidase activity was measured by following the oxidation of reduced cytochrome *c* at 550 nm[11]. 5'-Nucleotidase activity was measured according to BODANSKY AND SCHWARTZ[12] and glucose-6-phosphatase, acid phosphatase and phosphodiesterase according to DE LAMIRANDE *et al.*[13].

The concentration of protein was determined by the Lowry method using bovine serum albumin for reference standard[14].

Materials

Cytochrome *c*, 5'-AMP and glucose 6-phosphate were obtained from Sigma Chemical Co.

RESULTS AND DISCUSSION

The advantage in the use of an isotonic saline solution lies in its ability to pack the nuclei at low speed centrifugation while leaving behind plasma membranes and other membranous elements in suspension. The membranes are then pelleted by high speed centrifugation and separated on discontinuous sucrose density gradient centrifugation. A photograph showing the distribution of the different membrane fraction on a discontinuous sucrose density gradient is shown in Fig. 1. Three bands are

formed, one at each of the sucrose density interface. The amount of material in the bands varies depending on the mediums used to suspend the nuclei fraction containing membranes. Calcium containing medium gave a weak band whereas EDTA addition to isotonic NaCl provides a band containing more material. NaCl alone gave a band of intermediate size.

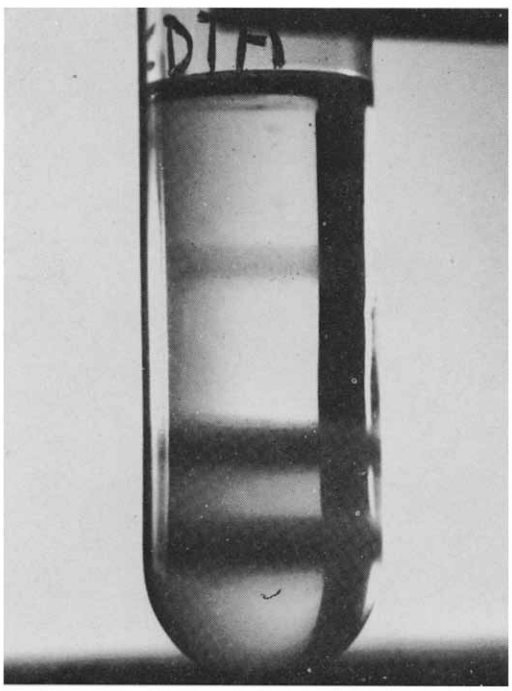

Fig. 1. Photograph showing the distribution of bands on discontinuous sucrose density gradient. Details are provided in the text.

Electron microscopy of the three bands showed that they consisted largely of membranous material. The top band arriving at 1.16–1.18 sucrose density interface was mostly composed of plasma membranes and their fragments with various configurations (Figs. 2A–2C). Occasionally, tight junctions, a feature of the plasma membrane in the tissue were observed. The basal surface (Disse's sinusoidal surface) of hepatocytes, covered by numerous microvilli representing a large portion of the surface area, was recovered in this fraction. In rare sections, we could see fragments what appeared to be bile canaliculi. Only a small portion of the sample contained mitochondria and membranes bearing ribosomes. The middle band arriving between 1.18–1.20 sucrose density interface contained considerable amounts of membrane vesicles with attached ribosomes besides having fragments of plasma membranes, whereas the last band of membranous material was predominantly contaminated by mitochondria and cell fragments. The sediment was composed of nuclei, mitochondria and cell fragments. It was also observed that the appearance of the plasma membranes (top band) in the electron microscope was similar when either of the three mediums were used for their elution from the nuclear fraction containing membranes. In the

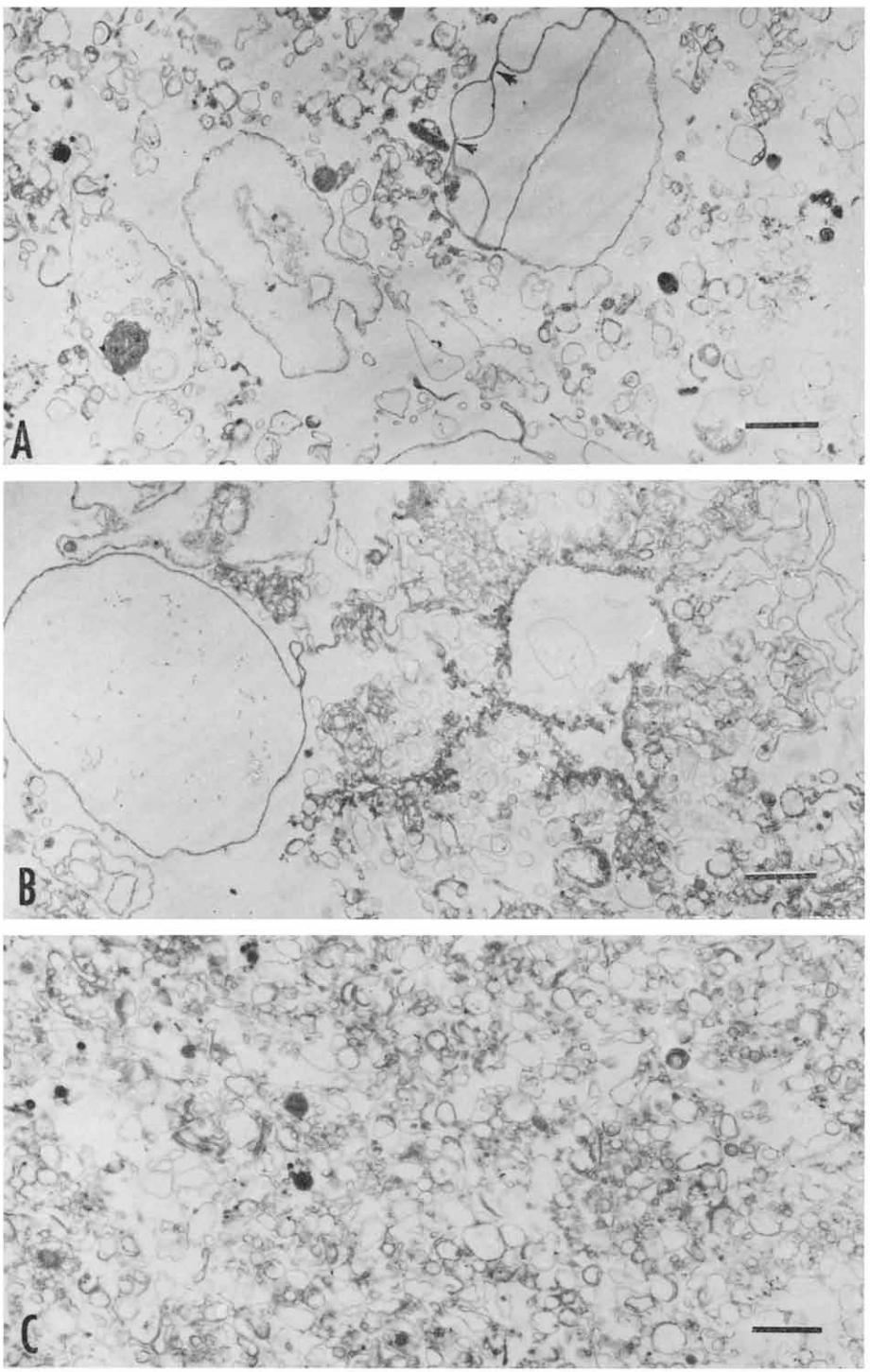
A
B
C

TABLE I

ENZYME ACTIVITIES IN RAT LIVER PLASMA MEMBRANE FRACTIONS

The rat liver plasma membranes were eluted from the nuclei containing membrane fractions with isotonic NaCl. Values for 5′-nucleotidase and glucose-6-phosphatase are in μmoles of phosphate liberated per h per mg of protein; in nmoles of phosphate liberated per h per mg of protein for the acid phosphatase; in nmoles of *p*-nitrophenyl-5′-thymidine phosphate hydrolyzed per min per mg of protein for phosphodiesterase; in nmoles of cytochrome *c* oxidized per min per mg of protein for cytochrome oxidase. Values are given as mean ± S.E. Number in parentheses indicates the number of preparations analyzed. —, not tested.

Cell fraction	*Phospho-diesterase* (4)	*5′-Nucleotidase* (8)	*Glucose-6-phosphatase* (5)	*Cytochrome oxidase* (5)	*Acid phosphatase* (4)
A. Homogenate	18 ± 0.72	3.92 ± 0.72	5.36 ± 0.68	300 ± 3.5	243 ± 5.1
B. Plasma membranes	132 ± 1.65	45.70 ± 3.30	1.82 ± 0.50	49 ± 4.0	97 ± 14.1
C. Mitochondria	—	—	—	950 ± 12.0	—
D. Microsomes	—	—	24.90 ± 1.30	—	—
E. Lysosomes	—	—	—	—	1010 ± 15.2
Ratio	B/A 7.3	B/A 11.7	B/D 0.1	B/C 0.05	B/E 0.09

presence of calcium and when isotonic NaCl alone was used, a certain amount of cytoplasmic matrix material adhering to plasma membranes was observed, although both mediums well preserved the tight junctions.

Table I gives data for the activities of five different enzymes in the plasma membrane fraction (top band), in the homogenate, and in different subcellular particles. Phosphodiesterase and 5′-nucleotidase (marker enzymes for liver plasma membranes) show, respectively, 7- and 12-fold enrichment over the liver homogenate. The mitochondrial enzyme cytochrome oxydase has a relative specific activity of 0.05 or 5% contamination of the plasma membranes with mitochondria. Similarly, a relative specific activity of 0.1 for glucose-6-phosphatase and acid phosphatase indicates a contamination of 10% by microsomes and lysosomes, respectively. Comparable enzymatic activities are obtained with the plasma membranes extracted by either isotonic saline, isotonic saline containing 0.5 mM $CaCl_2$ or isotonic saline containing 2 mM EDTA.

The plasma membrane protein yield varies depending of the medium used for elution; with isotonic saline, an average of 0.37 mg plasma membrane protein per g wet weight rat liver (8 different preparations) is recovered. The specific activity of these enzymes as well as protein recovery are similar to those reported by others[2,6,8].

Fig. 2. Electron micrographs of a cross-section of a pellet collected from the top band of discontinuous density gradient solution of sucrose. Marker line, 1 μm. A. Upper portion of the pellet contains vacuolated sheets which appear to be plasma membranes. The long sheets of plasma membranes of adjacent hepatocytes are seen interconnected by tight junctions (arrows). B. Middle portion of the pellet also contains large fragments of plasma membrane. The surface area facing Disse sinusoidal space appears to be isolated as groups of microvilli. The fibrillar cytoplasm which surrounds the border *in situ* and extends into their microvilli are remained immediately adjacent to the border. C. Lower portion of the pellet shows collections of vesicles and strips of membranes of relatively smaller sizes. Membrane vesicles with attached ribosomes are rarely present. × 10000.

CONCLUSION

We have shown in these studies that isoosmotic sucrose can be used to homogenize rat liver and to prepare a nuclear fraction containing plasma membranes. The post-nuclear supernatant fraction obtained can be further used to prepare by the classical centrifugation technique[15] the liver cell organelles.

Furthermore, the studies show that plasma membranes can be eluted from the nuclear fraction with isotonic saline solutions which protect nuclei against breakage, thus eliminating the obtention of plasma membrane fractions contaminated by nuclei membranes. It has been our experience that this method is reproducible with other rat strains.

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