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A MODIFIED METHOD FOR THE ISOLATION OF THE PLASMA MEMBRANE FROM RAT LIVER

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SUMMARY

A modified method for the isolation of the plasma membrane from rat liver has been developed. The concentration of Ca^{2+} and the degree of dilution of the homogenizing medium are critical factors in obtaining membranes in high yield and with high activity of the characteristic plasma membrane enzymes. The purity of the membranes was also checked by electron microscopy. Chemical analyses of the membranes are given.

INTRODUCTION

The plasma membrane plays a very important regulatory role in cellular metabolism and function. The method of isolation of plasma membrane originally developed by NEVILLE[1] and later modified by EMMELOT *et al.*[2–4] made possible the study of its biochemical properties[5–10]. However, these methods give a low yield of membrane. Other workers[11–13] later developed methods for isolation of the plasma membrane in isotonic medium. In some of these methods the membrane yield is increased but the activities of the functional enzymes are comparatively low.

Recent studies[14–16] on the nerve membrane have revealed that Ca^{2+} plays a key role in the maintenance of structural and functional integrity of the membrane. The stabilization of membrane structure by Ca^{2+} is believed to take place by way of coordination of Ca^{2+} to the macromolecular components of the membrane. For the preparation of liver cell suspension it has been found[17,18] that perfusion of liver with Ca^{2+}-free medium containing hyalouronidase and collagenase prior to disintegration of the tissue helps to make intact viable cells. Ca^{2+} is believed[19] to hold the cells together by forming a bridge between the surface membranes of the cells. The well known importance of Ca^{2+} in the reaggregation of dissociated embryos demonstrates the importance of this ion in establishing cell contact.

The object of the present investigation was to develop a suitable method which would enable one to prepare the plasma membrane from rat-liver tissue in higher yield and with higher activity of the characteristic membrane-bound enzymes.

MATERIALS AND METHODS

Protein was assayed by the method of LOWRY *et al.*[20] with bovine serum albumin as standard. Inorganic phosphorus was assayed by the method of Fiske and SubbaRow. Phospholipids were extracted with chloroform–methanol (2:1, by vol.) at room temperature. Phospholipid phosphorus was measured using the method of SHIN[21]. RNA was assayed with the orcinol reagent[22]. Sialic acid was determined by the method of WARREN[23].

Alkaline *p*-nitrophenyl phosphatase was assayed at pH 9.0 using *p*-nitrophenyl phosphate as substrate. The incubation mixture consisted of 100 μmoles Tris, 2.5 μmoles $MgCl_2$, 1.0 μmole *p*-nitrophenyl phosphate, 0.1 % Triton X-100 and enzyme in a total volume of 1.0 ml. The change in absorbance at 420 mμ was recorded using a Beckman D-B spectrophotometer.

Phosphodiesterase was assayed according to the method of OSTROWSKI AND TSUGITA[24] using bis-(*p*-nitrophenyl) phosphate as substrate.

5'-Nucleotidase was assayed at pH 7.5 and 10.0 in presence of 10 μmoles $MgCl_2$ using 5'-AMP as substrate. The incubation mixture consisted of 50 μmoles Tris, 10 μmoles $MgCl_2$, 10 μmoles AMP and 30–40 μg membrane protein in a total volume of 1.0 ml. After 10 min incubation the reaction was stopped by putting in boiling water bath for 2 min. The adenosine formed was separated by one dimensional paper chromatography using isobutyric acid–conc. NH_4OH–water (66:1:33, by vol.) as solvent. The spots were eluted with 1 M NH_4OH and the absorbance at 262 mμ was measured spectrophotometrically.

Glucose-6-phosphatase was assayed in 0.1 M acetate buffer (pH 6.1) at 30° using glucose 6-phosphate as substrate. The P_i liberated was assayed by the method of Fiske and SubbaRow.

Mg^{2+}-activated ATPase was assayed at pH 7.5 and at 37° using [^{3}H]ATP as substrate. The incubation system consisted of 10 μmoles Tris, 10 μmoles $MgCl_2$, 30–40 μg membrane protein and 10 μmoles ATP in a total volume of 1.0 ml. The ADP formed was separated by one dimensional paper chromatography using the isobutyric acid–NH_4OH–water solvent system. The spot of ADP was cut, put in a counting vial, 1 ml of water was added followed by 15 ml Bray's scintillation fluid. Radioactivity was determined with a Packard Tri-Carb scintillation counter.

Adenosine triphosphate pyrophosphohydrolase was assayed according to the method of LIEBERMAN *et al.*[8].

Adenyl cyclase was assayed as described previously[25].

RESULTS

The method of isolation of the plasma membrane is essentially similar to that used by NEVILLE[1] except that two steps have been included to improve the yield. (1) The homogenizing medium contained 0.5 mM $CaCl_2$ in addition to 1 mM HCO_3^-, the pH was adjusted to 7.5. (2) After homogenization, the homogenate was diluted 100-fold the wet weight of starting liver. Both the steps are important for increasing the yield of plasma membrane as determined by the recovery of the marker enzyme alkaline *p*-nitrophenyl phosphatase.

The membranes were isolated under ice-cold conditions according to the fol-

lowing procedure: 2-4 g of liver were homogenized in 50 ml homogenizing medium, mentioned above, in a Dounce homogenizer (loose pestle) using 25 gentle strokes. The homogenate was diluted 100 times the wet weight of fresh liver and allowed to stand for 5 min with occasional shaking. The diluted homogenate was then passed through four layers of fine cheesecloth (120 gauge). The filtrate was poured into plastic cups (250-ml capacity) and centrifuged at 2600 rev./min for 30 min in an International PR_2 refrigerated centrifuge. The supernatant was discarded and the pellet (Pellet 1) was suspended in the same buffer and homogenized gently (4–5 strokes). The suspension was diluted to half the previous volume and centrifuged again for 15 min at 2400 rev./min. The supernatant was discarded and the pellet (Pellet II) was resuspended by homogenization in half the previous volume of buffer and centrifuged again for 15 min. The supernatant was discarded and the pellet (Pellet III) was taken up in a small volume of buffer (1–3 ml) and then mixed with 70 % ice-cold sucrose solution (w/w) so that the final concentration of sucrose was 48 % (w/w). This sucrose suspension was divided equally in three of the Spinco SW-25.1 plastic tubes. Over each suspension was layered 8 ml 45 % sucrose, then 10 ml of 41 % sucrose (w/w) and finally 3–4 ml of 37 % sucrose (w/w). The tubes were then spun for 2 h at 25000 rev./min. The plasma membrane layer appears at the interface between 37 % and 41 % sucrose in the form of a thin compact sheet. The membrane layer was taken out with the help of a Pasteur pipette, washed free of sucrose and then suspended in an appropriate amount of buffer. This purified plasma membrane preparation has high enzymatic activities. Preparations were checked by electron microscopy. Nuclei, mitochondria, lysosomes, rough microsomes, Golgi membranes and collagen fibers were absent from most of the preparations. On rare occasions very few mitochondria and collagen fibers were observed. These contaminants could be removed by laying the membrane suspension on a continuous sucrose gradient and spinning for 5 h at $100000 \times g$. The membrane layer appears in the region of sucrose density 1.16. The membrane obtained by this method is very clean and devoid of any visible contaminants. A typical electron micrograph of the plasma membranes isolated by this method is presented in Fig. 1.

The importance of Ca^{2+} in increasing the yield of plasma membrane is shown in Table I. The recovery of the marker enzyme, alkaline *p*-nitrophenyl phosphatase in each of the particulate fractions and in the plasma membrane preparations is given. It can be seen that increasing the Ca^{2+} concentration to 1.0 mM, gives no further

TABLE I

RECOVERY OF ALKALINE *p*-NITROPHENYL PHOSPHATASE IN DIFFERENT FRACTIONS IN PRESENCE AND ABSENCE OF Ca^{2+}

Each figure represents the average of four determinations.

Fraction	*Percent of total alkaline p-nitrophenyl phosphatase activity*		
	No Ca^{2+}	*0.5 mM Ca^{2+}*	*1.0 mM Ca^{2+}*
Total homogenate	100	100	100
Pellet I	25	70	80
Pellet II	18	60	65
Pellet III	10	50	50
Plasma membrane	3	32	35

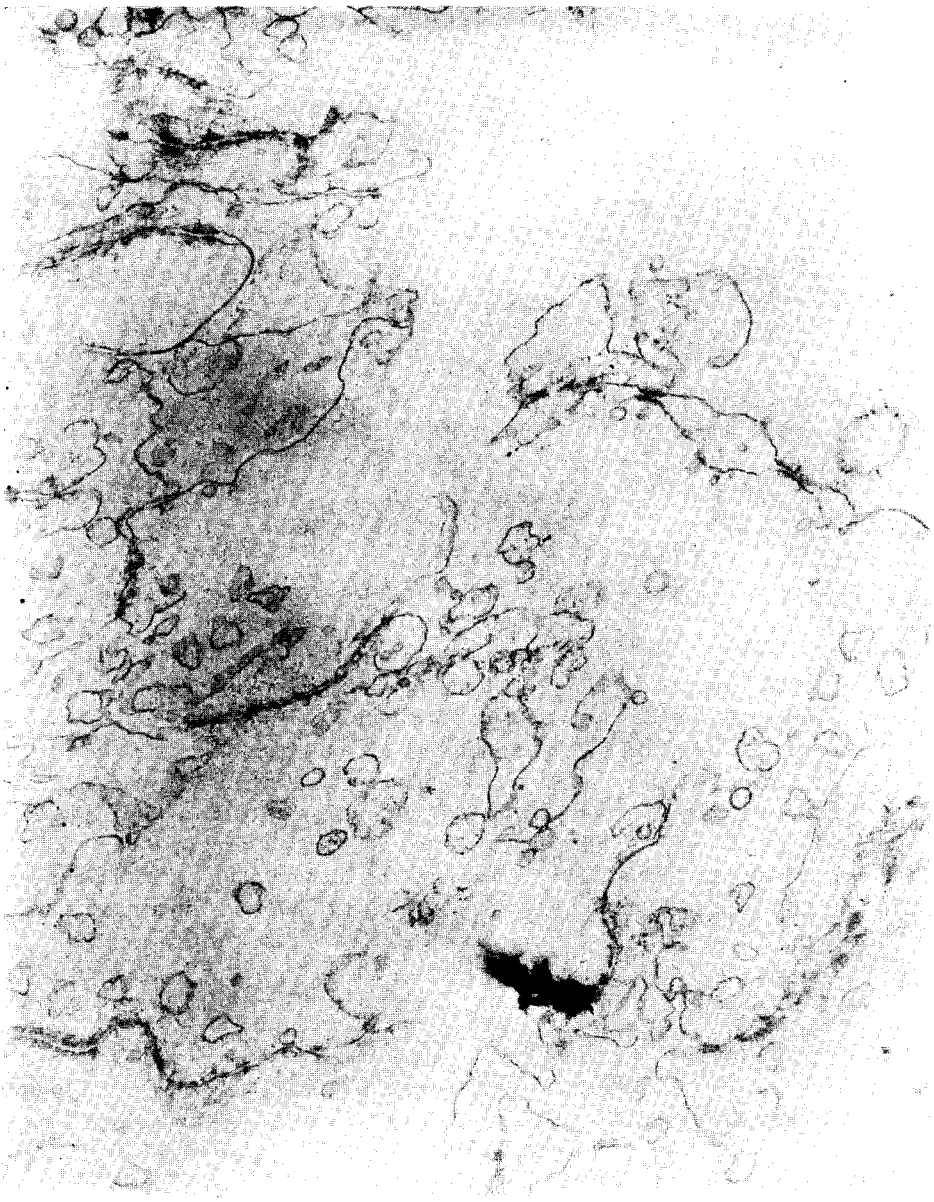

Fig. 1. Electron micrograph of isolated plasma membrane preparation. Membranes were fixed in 1 % OsO_4 (magnification × 25 000). The author is thankful to Mr. James V. Apicella for his technical assistance.

TABLE II

RECOVERY OF ALKALINE *p*-NITROPHENYL PHOSPHATASE IN PELLET I AND THE PLASMA MEMBRANE AFTER DIFFERENT DILUTIONS OF THE HOMOGENATE

Each figure represents the average of four determinations.

Extent of dilution	*Percent of total alkaline p-nitrophenyl phosphatase recovered*			
	With 0.5 mM Ca^{2+}		*Without Ca^{2+}*	
	Pellet 1	*Plasma membrane*	*Pellet 1*	*Plasma membrane*
1:25	40	18	15	0.9
1:50	60	25	20	1.2
1:100	70	32	30	3.0
1:200	70	30	35	3.0

TABLE III

CHEMICAL COMPOSITION OF THE PLASMA MEMBRANE

Each figure represents an average of four determinations. The numbers in parentheses give the range of values.

Composition	*Present method*	EMMELOT *et al.*[5]	COLEMAN *et al.*[13]
mg protein per g wet liver	1.3 (1.0–1.5)	0.41	1.3 (0.87–2.3)
μg total P per mg membrane protein	26.0 (24–29)	12.5	20.6
μg phospholipid P per mg protein	24.3 (22.5–25.0)	11.25	*
μg RNA per mg protein	10.0 (8–12)	*	*
μg sialic acid per mg protein	16.0 (14.2–18.5)	9.7	*

* Not given in the references.

TABLE IV

ENZYME ACTIVITIES OF THE PLASMA MEMBRANE AND SMOOTH MICROSOMES

The numbers in parentheses represent number of experiments done, except in the third column where the range of values is given.

Enzyme	*Specific activity (μmoles/mg protein per h)*			
	Present method		COLEMAN *et al.*[13]	EMMELOT *et al.*[5]
	Plasma membrane	*Smooth microsome**	*Plasma membrane*	*Plasma membrane*
Alkaline *p*-nitrophenyl phosphatase	8.0 (20)	0.5 (20)	**	0.92 ± 0.28
Adenosine triphosphate pyrophosphohydrolase	70 (20)	7.4 (20)	**	**
Phosphodiesterase	1.1 (10)	0.07 (10)	**	**
5′-Nucleotidase (pH 7.5)	82.0 (4)	15.0 (3)	58.6 (31.3–91.1)	32.2 ± 6.8 (9)
5′-Nucleotidase (pH 10.0)	150.0 (4)	30.0 (3)	**	**
Mg^{2+}-activated ATPase	200.0 (3)	**	23.0 (8.5–39.3)	44.2 ± 6.4 (12)
Adenyl cyclase	0.085 (5)	Very low	**	**
Glucose-6-phosphatase	1.25 (20)	22.0 (20)	3.05 (0.78–6.9)	1.44 ± 0.24 (4)

* Smooth microsome was prepared by the method of DALLNER *et al.*[26].

** Not given in the references, or not determined.

increase in the recovery of alkaline phosphatase and that 0.5 mM Ca^{2+} seems to be the optimum concentration.

Dilution of the homogenate is also another important factor for enhancing the yield of plasma membrane as is evident in Table II.

Chemical composition of plasma membrane

The yield and chemical composition of the plasma membrane are given in Table III.

By using the optimum conditions of 0.5 mM Ca^{2+} and 1:100 dilution, the yield of plasma membrane was 1.0–1.5 mg of plasma membrane protein per g wet liver as compared to 0.41 mg membrane protein per g wet liver by the method of NEVILLE[1] and 0.87–2.3 mg membrane protein per g liver by the method of COLEMAN *et al.*[13].

Enzymatic composition of plasma membrane

The activities of different enzymes present in plasma membrane are shown in Tabel IV. The specific activities of enzymes in smooth microsomes are also presented in the table in order to show the relative distribution of the enzymes in the two subcellular structures.

DISCUSSION

It appears that when the liver tissue is homogenized in absence of Ca^{2+}, the amount of Ca^{2+} present originally in the plasma membranes is to a great extent dissociated from the membrane structure. As a result of this loss of Ca^{2+}, the membrane becomes less dense and fragile and fails to sediment at the same position as the more intact membranes in the sucrose gradient. The presence of Ca^{2+} in the external medium may help to prevent the dissociation of the membrane-bound Ca^{2+} and in this way may help to keep the plasma membrane more intact, thereby resulting in greater yield. The electron micrographs of the plasma membrane preparations prepared in absence and presence of Ca^{2+} showed that the membranes prepared in presence of Ca^{2+} were more intact and contained longer continuous structures and less vesicles.

In the vertebrate liver about 80 % of the total cell population are parenchymal cells[27,28]. Plasma membranes of the adjoining parenchymal cells make up bile canaliculi, which can be readily identified by means of electron microscopy[29–32]. As the 'bile fronts' are important parts of the plasma membranes of the adjoining hepatocytes, and alkaline phosphatase is known to be associated with the bile canaliculi, this enzyme was used as the marker enzyme for the isolation of plasma membranes.

The effect of dilution of the homogenate on the increased recovery of the enzyme may be due in part to a lowering of the viscosity of the homogenate and consequently more of the membranes become sedimentable at the particular centrifugal force used for their isolation.

As seen in Table II, using 0.5 mM Ca^{2+} and 1:100 dilution, 70 % of the total *p*-nitrophenyl phosphatase activity can be recovered in Pellet I. The rest of the activity fails to sediment possibly due to extensive fragmentation of the plasma membrane of the cells during homogenization. In the two subsequent steps of washing of the Pellet I, 20 % of the total activity is again lost. This loss also seems to be due

to fragmentation of the membranes during resuspension of the pellet by homogenization. Out of the remaining 50 % of the total *p*-nitrophenyl phosphatase activity in Pellet III, 32 % of the total can be recovered in the plasma membrane fraction, the rest was found to be distributed throughout the sucrose gradient. During isolatation of the membranes in sucrose gradient the membranes experience some osmotic shock, which seems to be the cause of dispersion of some of the membrane proteins. This was also observed when the membranes were put on the continuous gradient for further purification. The continuous gradient does not only remove the undesirable trace contaminants from the plasma membrane, but also at the same time causes a change in some of the membrane-bound proteins like alkaline *p*-nitrophenyl phosphatase and adenosine triphosphate pyrophosphohydrolase, such that they distribute themselves throughout the gradient. The low activity of *p*-nitrophenyl phosphatase in microsomal fraction may be due to contamination of microsomes by tiny fragments of plasma membranes. Assuming that most of the *p*-nitrophenyl phosphatase activity of the liver cells is associated with the plasma membranes the theoretical yield of plasma membranes should be about 4.0 mg membrane protein per g of wet liver tissue (*i.e.*, about 3.3 % of the total protein of the cell). On the basis of this calculation the yield of plasma membrane by this method is about one-third the theoretical yield.

Table III shows the chemical composition of the plasma membrane prepared by this method compared to those of other methods. The phosphorus/protein ratio of the membrane preparations are much higher than that observed by EMMELOT *et al.*[5] but very close to the values of COLEMAN *et al.*[13] and DOD AND GRAY[6]. The sialic acid/protein ratio is also higher in the present preparation. The membranes isolated in absence of Ca^{2+} have a faint pinkish color, possibly coming from a contamination by haemoglobin. This colored protein is soluble in saline. The membranes prepared in presence of Ca^{2+} were not found to have this colored protein. The data presented in this paper resemble more closely the saline-insoluble fraction of EMMELOT *et al.*[5].

The specific activities of characteristic plasma membrane enzymes (Table IV) are higher in membranes prepared by this method. The relative activities of those enzymes present in smooth microsome are also presented. Glucose-6-phosphatase is considered to be a microsomal enzyme. The low activity of glucose-6-phosphatase in these plasma membrane preparations may suggest contamination of the preparation by smooth microsome to the extent of 4–5 %.The presence of higher glucose-6-phosphatase activities in membranes prepared by COLEMAN *et al.*[13] may suggest higher degree of contamination by smooth microsomes. The specific activities of 5′-nucleotidase and Mg^{2+}-activated ATPase are much higher in the present membrane preparations compared to the membranes prepared by other methods[5,13]. The 5′-nucleotidase activity of the membrane preparations exhibited two optimum pH's, one at pH 7.5 and another at pH 10.0 in the presence of 10 mM $MgCl_2$. The double pH optimum for 5′-nucleotidase was also observed by SONG *et al.*[33] in rat-liver cell plasma membranes and by other workers in rat[34,35] and human[36] livers. This pronounced activation by Mg^{2+} at pH values near 10.0 seems to be characteristic of 5′-nucleotidase from those sources and the kinetics of such activation have been discussed by LEVIN AND BODANSKY[37]. In the membranes prepared by the present method the 5′-nucleotidase activity at pH 10.0 was always higher than at pH 7.5.

In this connection it is noteworthy that the 5′-nucleotidase activity of plasma membrane is greatly inhibited in presence of 4–5 mM concentration of ADP[38].

TAKENCHI AND TERAYAMA[12] used isotonic sucrose solution with 0.5 mM Ca^{2+} as the homogenizing medium for preparing liver cell plasma membranes. The present method differs from the method of TAKENCHI AND TERAYAMA[12] by the use of hypotonic bicarbonate buffer and by the degree of dilution of the homogenate. The yield of plasma membrane reported by TAKENCHI AND TERAYAMA[12] seems to be much lower compared to the present method.

The present method has been used to study the biosynthesis of membrane components *in vivo*[39].

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