Biochimica et Biophysica Acta, 332 (1974) 145–155
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 76541

HIGHLY PURIFIED PLASMA MEMBRANES FROM RAT HEPATOCYTES FOLLOWING RATE–ISOPYCNIC ZONAL CENTRIFUGATION

D. GAVARD, G. DE LAMIRANDE and S. KARASAKI

Institut du Cancer de Montréal, Hôpital Notre-Dame and Départements de Biochimie et d'Anatomie, Université de Montréal, Montréal (Canada)

(Received September 7th, 1973)

SUMMARY

Plasma membranes from liver parenchymal cells were isolated by rate–isopycnic zonal centrifugation. A method is described for the Beckman size 15 zonal rotor. It involved preparation from a perfused liver of a parenchymal cell-enriched homogenate in isoosmotic sucrose. The nuclear fraction containing membranes was recovered by centrifugation. The resuspended pellet was applied on the gradient of the zonal rotor. The isolated membranes had the same isopycnic banding density as 37% sucrose (w/w). The specific activity of 5′-nucleotidase, a widely used plasma membrane marker, was 105 μmoles·(mg protein)$^{-1}$·h^{-1} being enriched by a factor of 50 as compared with parenchymal cell homogenate. The plasma membrane fraction was free of the mitochondrial and lysosomal enzymes, succinate dehydrogenase and acid phosphatase. No DNA and 10 μg RNA per mg plasma membrane protein were found. The purity of the membranes and their morphological appearance were controlled by electron microscopy. The preparation consisting of large membrane sheets showed a considerable purification away from other cellular components. A comparison with similar methods indicates that plasma membranes of a higher degree of purity can be obtained from parenchymal cells.

INTRODUCTION

The method of isolation of plasma membrane originally developed by Neville [1] and later modified by Emmelot and Bos [2,3] made possible the study of its biochemical properties [4–7]. However, this procedure involves homogenization of liver in ice-cold water buffered with 1 mM $NaHCO_3$. Because of the possibility that water might alter the physical structure or biochemical properties of the membranes, similar isolations were attempted by various groups starting with liver homogenates made in isotonic sucrose [8–10]. Attempts to prepare plasma membranes from perfused livers or isolated cells, using the above methods have been reported to be unsuccessful [11,12].

The present method represents a mild procedure for the isolation of plasma membranes from liver parenchymal cells in order to preserve structural components

Abbreviation: INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

and enzymatic activities. A parenchymal cell-enriched homogenate was prepared from a liver in which cell contact has been lowered by perfusion with a Ca^{2+}-free medium. The 5'-nucleotidase was chosen as the indicator enzyme for the plasma membrane fragments because this enzyme has been visualized cytochemically and appears to be present only in the plasma membranes of the parenchymal cells [13, 14]. A nuclear pellet, containing much of the nucleotidase of the homogenate was used as the starting material for the rate–isopycnic zonal centrifugation. The use of a large zonal rotor has the advantage of reducing the repeated washings of the nuclear fraction in order to remove small contaminating organelles which are common features of all non-zonal methods.

The present procedure gives a preparation of plasma membranes of a high degree of purity as judged by biochemical and morphological criteria. The utility of a pure preparation for the study of enzymes in the plasma membranes of rat liver cells, as well as for other studies such as membrane structure is obvious.

MATERIALS AND METHODS

Preparation of the crude nuclear fraction

Wistar male rats of an average weight of 250 g were sacrificed by decapitation after a fasting period of 18 h. Livers were perfused in situ through the portal vein with Ca^{2+}-free Hank's solution [15] containing 38 mM sodium citrate. The perfused livers were excised and put into the same ice-cold solution. Homogenization and fractionation were carried out at 4 °C.

A 10% liver homogenate in 0.25 M sucrose, $NaHCO_3$ (pH 7.6) was first prepared using a Potter–Elvehjem homogenizer. This homogenate containing connective tissue and other non-parenchymal cells was used to obtain values for the whole liver.

For the preparation of purified membranes, the homogenate was prepared as follows: 8 g of liver were homogenized by hand in 80 ml of the homogenization medium mentioned above with a Chauveau homogenizer (Chauveau, J., personal communication). The homogenization was pursued until more than 90% of the parenchymal cells were broken. The homogenate was then filtered through two sieves with mesh of 250 and 44 μm respectively. Quantitative histological examination of the homogenate on smears, as previously described [16] showed the presence of less than 5% of non-parenchymal cells.

The homogenate was centrifuged at 400, 1000 or $1720 \times g$ for 10 min. The resulting nuclear pellet was suspended in about 40 ml of the homogenization medium with two strokes of a Potter–Elvehjem homogenizer (clearance 13/1000 inch) with the pestle rotating at 1000 rev./min.

Isolation of plasma membranes by rate–isopycnic zonal centrifugation

Using a Beckman 141 high capacity gradient pump, a 1200-ml linear density gradient ranging from 20 to 35% sucrose (w/w) was introduced into a Beckman Ti-15 zonal rotor spinning at 3000 rev./min in a Beckman L3-50 centrifuge. This was followed by 300 ml of 45% sucrose (w/w) and then by 165 ml of 55% sucrose (w/w). A 40-ml sample of the $400 \times g$ sediment resuspended in the homogenization medium was then applied on the gradient followed by an overlay of 80 ml of 0.08 M sucrose.

TABLE I

RATE-ISOPYCNIC CENTRIFUGATION SYSTEM FOR THE PLASMA MEMBRANE PREPARATION OF PARENCHYMAL CELLS

Rotor Beckman Ti-15. Stage 1: Rate separation at 3000 rev./min for 25 min; eluted 480–560 ml at 3000 rev./min. Stage 2: Isopycnic banding at 20000 rev./min for 60 min; eluted at 3000 rev./min.

Component	Solution	Volume (ml)
Overlay	0.08 M sucrose, $NaHCO_3$ (pH 7.6)	80
Sample	Crude nuclear fraction in 0.25 M sucrose, $NaHCO_3$ (pH 7.6)	40
Gradient	20–35% sucrose (w/w), $NaHCO_3$ (pH 7.6)	1200
First interface	45% sucrose (w/w), $NaHCO_3$ (pH 7.6)	300
Cushion	55% sucrose (w/w), $NaHCO_3$ (pH 7.6)	to fill rotor

This is summarized in Table I. All the sucrose solutions were buffered at pH 7.6 with $NaHCO_3$ as well as the homogenization medium.

Following the injection of the sample material, the centrifugation was continued for 25 min to achieve a rate separation. Twelve to fourteen 40-ml fractions were then removed from the rotor by pumping 55% sucrose (w/w) and a 80 ml-overlay was added. The rotor speed was increased to 20000 rev./min for 1 h to achieve isopycnic banding. The entire contents of the rotor were then unloaded after the rotor has been decelerated to 3000 rev./min.

Density was calculated from the refractive index of each fraction measured with an Abbe refractometer. The absorbance of the fractions was determined at 260 and 280 nm using a Hitachi-Perkin Elmer 360 spectrophotometer. The plasma membrane containing fractions were diluted with an equal volume of deionized water. They were then concentrated by centrifugation at $20000 \times g$ for 20 min in the Beckman No. 30 rotor. Collected pellets (called sedimented fractions) were stored at 0 °C overnight.

Marker enzymes

Among the markers employed in establishing purity, enzymes are most widely used for reasons of sensitivity, precision and quantitation. Moreover, they link in vitro fractionations to in situ histochemical evidence.

The results of the assays which were performed directly on the fractions derived from the gradient were corrected for sucrose interference [17]. Correction curves have been established for protein and each enzyme studied.

Enzyme activities were determined on the basis of 30 min of incubation with the appropriate substrate at 37 °C. An appropriate dilution of tissue fractions was made before enzyme analysis based on the range of activity which was expected in each fraction.

Determination of 5'-nucleotidase, glucose-6-phosphatase and acid phosphatase. For the assays involving the release of inorganic phosphate, reactions were terminated by the addition of cold trichloroacetic acid. The precipitate was removed by centrifugation at $10000 \times g$ for 10 min. Corrections were made for the presence of P_i formed in the absence of substrate and for the release of phosphate due to non-enzymatic

hydrolysis of substrate. 5'-Nucleotidase was used as a marker for the plasma membranes [14, 18]. Its activity was measured as described by Michell and Hawthorne [19] with sodium AMP as substrate. The inclusion of tartrate in the assay medium as an inhibitor of acid phosphatase was suggested by El Aaser and Reid [20]. Glucose-6-phosphatase, a marker for endoplasmic reticulum, was assayed by the method of Swanson [21]. Acid phosphatase, a marker for lysosomes, was estimated by the method of Gianetto and De Duve [22], after 4 freezings and thawings of the fractions, using 0.1 M β-glycerophosphate as substrate.

Determination of succinate dehydrogenase. Succinate dehydrogenase, a marker for the mitochondria, was assayed by measuring the formazan formed from reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) [23]. The formazan of INT was measured at 490 nm after extraction into ethyl acetate.

Determination of phosphodiesterase I. Phosphodiesterase I was determined in a total volume of 2.5 ml containing 0.7 mM *p*-nitrophenyl-5'-thymidylate and 60 mM Tris–HCl buffer (pH 8.8). Appropriate controls without enzyme or substrate were run concurrently. After 15 min, the reaction was stopped by the addition of 1.0 ml of 0.1 M NaOH. The protein precipitate was removed by centrifugation. Liberated *p*-nitrophenol in supernatant was measured at 400 nm and calculated using a molar extinction coefficient of 18200 $l \cdot mole^{-1} \cdot cm^{-1}$.

Chemical analyses

P_i was assayed by a modification of the method of Fiske and SubbaRow [24]. Protein was measured according to the method of Lowry et al. [25] with crystalline serum albumine as standard. The RNA content of the membranes was determined by the Schmidt–Thannhauser method as described by Munro and Fleck [26]. DNA was solubilized from the residue left after the extraction of the RNA with hot KOH and then estimated by the modification of Giles and Myers [27] of the Burton's procedure.

Electron microscopy

The identification and characterization of the isolated liver membranes was also based on their morphological and ultrastructural appearance which is so characteristic that it can serve as a reliable marker.

Every step of the fractionation was monitored by phase contrast microscopy. The membrane pellets obtained in the final centrifugation were fixed for 30 min in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 4% sucrose and 0.07 M $CaCl_2$. They were then washed in the buffer and were postfixed for 2 h in 1.3% collidine-buffered OsO_4 (pH 7.2). After dehydration in ethanol, the pellets were embedded flat in an Epon mixture. The complete cross sections of oriented pellets, 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.

Chemicals

Ribonuclease-free sucrose, AMP (sodium salt), glucose 6-phosphate (monosodium salt), β-glycerophosphate (disodium salt) and INT were purchased from Sigma; succinic acid (disodium salt) from Matheson; ethyl acetate from J. T. Baker,

and *p*-nitrophenyl-thymidine-5′-phosphate (sodium salt) from Calbiochem. All other chemicals used were of analytical grade.

RESULTS

Preparation of the crude nuclear fraction

A buffered isoosmotic sucrose solution was chosen for the preparation of the homogenate to protect the other cellular components such as mitochondria, lysosomes and nuclei from breaking. As indicated in Table II, about 47% of the total amount of 5′-nucleotidase was recovered in the pellet after 10 min centrifugation at $400 \times g$. This fraction was enriched with the marker for plasma membranes since the pellet contained only 36% of the proteins of whole homogenate. Little of the remainder of 5′-nucleotidase seemed to sediment at $1000 \times g$ or $1720 \times g$, whereas a further 13% of the original glucose-6-phosphatase and almost all of the succinate dehydrogenase sedimented at these higher speeds. Centrifugation for 10 min at $400 \times g$ therefore furnishes the sheets of membranes while giving minimal contamination by mitochondria. The $400 \times g$ supernatant still contained about 50% of the original 5′-nucleotidase activity, but this is known to reside in vesicles rather than sheets [20]. It was concluded from these results that further separation procedures using the $400 \times g$ pellet might be productive.

Sedimentation properties of plasma membranes from rat hepatocytes

A typical sedimentation profile obtained by the rate–isopycnic centrifugation (Table I) is shown in Fig. 1. The sample applied to the density gradient was the $400 \times g$ sediment prepared as described above from 8 g of rat liver. The contents of the rotor were removed in two stages by displacement with 55% sucrose (w/w), and fractions of 40 ml were collected.

A volume of 520 ml was removed after centrifugation of 25 min at 3000 rev./min. The displaced fluid included the mitochondria, lysosomes and endoplasmic reticulum contained in the sample introduced into the zonal centrifuge, as indicated by the various enzyme activities (Fig. 1 b). The greater part of the 5′-nucleotidase was also associated with the more slowly sedimenting particles (Fig. 1a).

After achieving isopycnic banding (20000 rev./min; 1 h) of the remaining

TABLE II

PREPARATION OF CRUDE NUCLEAR FRACTION

Values listed are the means of percentage of homogenate activity obtained for each fraction in 2 or 3 separate experiments.

Fraction	5′-Nucleotidase (%)	Succinate dehydrogenase (%)	Glucose-6-phosphatase (%)	Acid phosphatase (%)	Protein (%)
$400 \times g$ sediment	47	40	28	20	36
$1000 \times g$ sediment	53	95	32	—	50
$1720 \times g$ sediment	49	98	41	—	60

material, the membranes were collected as a sharp peak (Fig. 1a). The average banding density for 12 runs was approx. 1.16 g/ml at 20 °C. The yield of membranes by this procedure was about 5% of the 5'-nucleotidase of the homogenate. There was a 38-fold increase in specific activity of the 5'-nucleotidase in the sedimented membrane fraction as compared to the activity of this enzyme in the homogenate of parenchymal cells. The membranes were free of nuclei, mitochondria and lysosomes (Fig. 1b). They contained about 0.08% of the glucose-6-phosphatase of the homogenate. An amount of 10 μg RNA per mg membrane protein was detected in the plasma membrane fraction. No DNA was found to be present under conditions of the improved diphenylamine reaction as described by Giles and Myers [27].

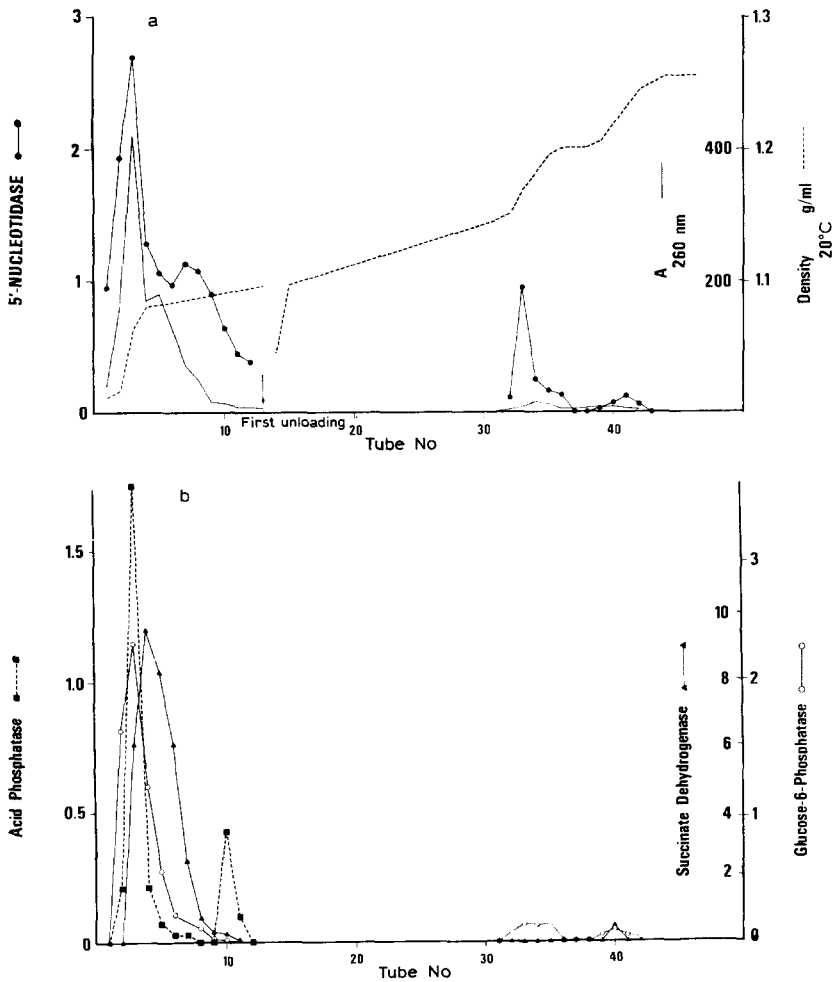

Fig. 1. Rate–isopycnic zonal fractionation of nuclear sediment obtained from isolated parenchymal cells in the Ti-15 rotor. Enzyme units are given as μmoles P_i or formazan per 40 ml per min. a. Absorbance, density and 5'-nucleotidase profiles of zonal fractions. b. Profiles of acid phosphatase, succinate dehydrogenase and glucose-6-phosphatase in zonal fractions.

There was also a small amount of 5′-nucleotidase found in zonal fractions containing predominantly nuclei (Tubes 40–41). Addition of total 5′-nucleotidase values for all of the zonal fractions gave a total recovery of 15 μmoles P_i released per min. Of this total, 0.3 μmole was attributable to structures having a density greater than 1.2 g/ml at 20 °C.

The sedimentation profile obtained for a crude nuclear fraction prepared from a whole liver homogenate was compared to the one obtained from a parenchymal cell enriched homogenate (Fig. 2). An additional band of 5′-nucleotidase activity was found (Fig. 2a). Phase-contrast microscopy showed this band to contain fragments of connective tissue. When the homogenate contained less than 5% of non-parenchymal

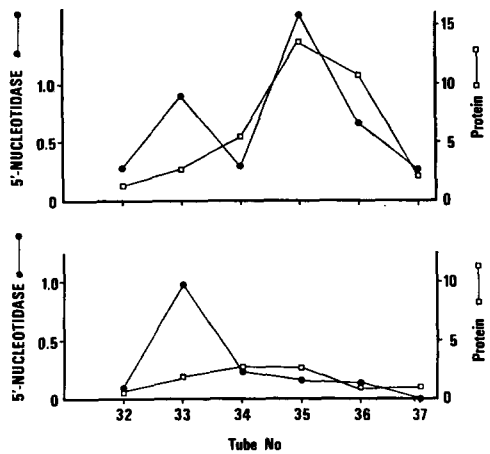

Fig. 2. Pattern of plasma membrane region showing the position of connective tissue. a. (Top) Crude nuclear fraction prepared from a whole liver homogenate. b. (Bottom) Crude nuclear fraction prepared from an homogenate containing less than 5% of non-parenchymal cells. Rate-isopycnic zonal separations were carried out as described in Table I. 5′-Nucleotidase activity is expressed as μmoles P_i per 40 ml per min. Protein assayed by the procedure of Lowry et al. [25] are given in mg per 40-ml fraction.

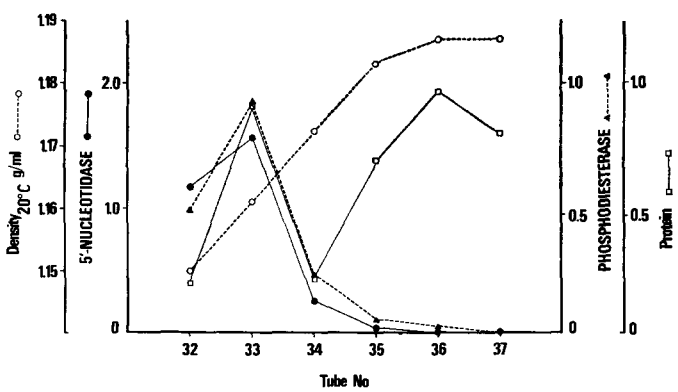

Fig. 3. Pattern of the plasma membrane region obtained with a first interface of 42.6% (w/w) sucrose. The specific activities for 5′-nucleotidase (μmoles P_i per mg per min) and for phospho-diesterase I (μmoles *p*-nitrophenyl-thymidine-5′-phosphate per mg per min) obtained for each tube is plotted. The protein are expressed as mg per 40-ml fraction.

cells, the band of 5′-nucleotidase associated with connective tissue disappeared (Fig. 2b). The purification of plasma membranes according to 5′-nucleotidase was 7-fold when whole liver homogenate was used as the starting material, whereas a 38-fold increase in specific activity was achieved with the enriched homogenate.

In order to improve the purification of the plasma membranes, it was decided to lower the first interface from 45% sucrose (w/w) to 42.6%. This should permit further migration in the gradient of the contaminating material, if any. As shown in Fig. 3, the proteins in the plasma membrane region can be fractionated in two bands by this new gradient form. A 50-fold purification according to 5′-nucleotidase is thus achieved in the plasma membrane fraction (Tube 33). It can also be seen from Fig. 3 that phosphodiesterase I activity, another reliable marker for the plasma membrane [18,28] closely followed 5′-nucleotidase activity in the gradient.

Distribution of marker enzymes

Table III gives data for the activities of five different enzymes in the homogenate of isolated parenchymal cells, in the crude nuclear fraction, in the zonal plasma membrane fraction and in the sedimented plasma membrane fraction. Phosphodiesterase I and 5′-nucleotidase (marker enzymes for liver plasma membranes) show, respectively, a 37- and 50-fold enrichment over the total homogenate in the sedimented plasma membrane fraction. The specific activity of the microsomal marker enzyme, glucose-6-phosphatase, is lower in the plasma membranes than in the homogenate. The yield of glucose-6-phosphatase in the plasma membrane represents only 0.08% of the homogenate and is significantly lower than that of the marker enzymes (5.1 and 3.3%). The activities of mitochondrial and lysosomal marker enzymes are not detectable in the plasma membrane fraction. The sensitivity of the assay for succinate dehydrogenase is such that an enzyme level more than 0.01% of the total found in the homogenate could be detected.

TABLE III

PURITY AND YIELD OF PLASMA MEMBRANE FRACTION

Each mean value is followed by it sstandard deviation and number of observations in parentheses. μmoles of phosphate liberated per h per mg of protein; in μmoles of *p*-nitrophenyl-5′-thymidine formed per h per mg of protein for succinate dehydrogenase.

	5′-Nucleotidase			Phosphodiesterase I		
	Spec. act.	Recovery (%)	Relative spec. act.	Spec. act.	Recovery (%)	Relative spec. act.
Homogenate	2.1 ± 0.8 (14)	100	1	4.1 ± 0.7 (8)	100	1
400 × *g* Sediment	2.7 ± 1.1 (12)	51.3 ± 6.1	1.29	6.2 ± 0.6 (7)	52.6 ± 4.7	1.5
Zonal plasma membrane fraction	44 ± 16 (5)	3.4 ± 1.5	21	128 ± 21 (7)	4.5 ± 1.0	31.2
Sedimented plasma membrane fraction	105 ± 13	5.1 ± 2.5	50	150 ± 30	3.3 ± 0.6	36.6

Morphology of isolated plasma membranes

Under the phase contrast microscope, the surface membranes obtained by this method were relatively large fragments up to 30 μm in size. Electron microscopy of the preparation demonstrated that the fraction was practically free from nuclei, mitochondria, lysosomes and microbodies. The fractions contained predominantly large sheets of plasma membranes, various smooth surfaced membranes forming microvilli, vesicles or flattened sacs, some of which enclosed electron-dense material. The plasma membranes were not contamined by fragments of rough endoplasmic reticulum and nuclear envelope, since membranes with attached ribosomes were absent. A considerable proportion of the membranes were seen paired and were occasionally interconnected by means of junctional complexes. Gap junctions, the surface specialization characteristic of the lateral membranes between adjacent hepatocytes, could be recognized in the intact form while desmosomes had a loosened appearance. Plasma membranes in the single sheets were often accompanied by numerous pinocytic vesicles.

DISCUSSION

It is well known that the mammalian liver contains a heterogeneous cell population, with hepatic parenchymal cells comprising approx. 65% of the total [29]. Attempts to prepare plasma membrane from an homogenate containing less than 5% of non-parenchymal cells, using a two-stage zonal centrifugation, yielded a preparation of a high degree of purity. This can be judged by the relatively high specific activity of the plasma membrane marker enzymes as well as by the absence of any succinate dehydrogenase or acid phosphatase activities. Values reported for the relative specific activity of 5'-nucleotidase in purified plasma membranes (ratio of the

Values for specific activity of 5'-nucleotidase, glucose-6-phosphatase and acid phosphatase are in phosphate hydrolyzed per h per mg of protein for phosphodiesterase; in μmoles of formazan

Glucose-6-phosphatase			Succinate dehydrogenase			Acid phosphatase		
Spec. act.	Recovery (%)	Relative spec. act.	Spec. act.	Recovery (%)	Relative spec. act.	Spec. act.	Recovery (%)	Relative spec. act.
2.6 ±0.3 (4)	100	1	4.5±0.9 (5)	100	1	1.99±0.16 (3)	100	1
2.2 ±0.8 (4)	38 ±8	0.84	5.3±1.6 (5)	41±4	1.17	1.3 ±0.3 (3)	17.3±2.1	0.65
—	—	—	0	0	—	0	0	—
1.54±0.2	0.08±0.04	0.59	0	0	—	0	0	—

specific activity of the plasma membrane preparation to the specific activity of the starting liver homogenate) vary from 12 to 30 [10, 12, 24, 30–33]. It must be taken into consideration that not all enzymatic assays were performed under identical conditions. However, the present method gave plasma membrane fragments which showed a 50-fold increase in 5′-nucleotidase specific activity over the homogenate. Hinton et al.[33], with a similar yield obtained a 30-fold increase in a preparation by zonal centrifugation of plasma membranes still possessing some succinate dehydrogenase and acid β-glycerophosphatase activities. These two enzymes were undetectable in our purified plasma membrane fraction.

Glucose-6-phosphatase is generally accepted as an indicator of contamination by the endoplasmic reticulum, although there is no definitive evidence to indicate that this enzyme or others which catalyze hydrolysis of glucose-6-phosphate are indeed absent from plasma membranes. To our knowledge, there is only one report on the preparation of plasma membranes essentially free of this enzyme activity [10]. Glucose-6-phosphatase was at a low level of activity in our membrane preparation. Its relative specific activity of 0.59 compared advantageously well with the values obtained by quite different procedures such as such as 0.49 by Hinton et al. [33], 1.4 by Emmelot and Bos [3] or 0.23–2.05 by the procedure of Coleman [10]. The low RNA content of the membranes, comparable to the lowest reported [34], as well as the absence under the electron microscope of any membranes with attached ribosomes are also suggestive of the absence of microsomal contamination.

Electron microscopy rendered evidence for a rather pure preparation of the plasma membranes of hepatocytes which was not contaminated by other identifiable cellular components. Complete absence of collagen fibers and basement membrane material may suggest that the plasma membranes arise from hepatocytes. It was possible to identify all surface membrane specializations, i.e. canalicular, lateral and sinusoidal surfaces. The techniques used for perfusion and homogenization seemed to have partially damaged some portions of the canalicular and/or sinusoidal surfaces. However the damage is rather slight since microvilli and pinocytotic vesicles still attached to the membrane sheets are recovered. This is in agreement with the observed high specific activity of 5′-nucleotidase which is characteristic of these surface specializations. Undoubtedly, the lateral membranes isolated from hepatocytes could easily be identified in the electron microscope on the account of the presence of junctional complexes between membranes. It may be of interest that gap junctions have been well preserved although desmosomes often had a loosened appearance.

Weaver and Boyle [23] also used a two-step zonal centrifugation for purifying plasma membrane with a B-XV zonal rotor. The present method differs from the method of Weaver and Boyle by the use of buffered isoosmotic sucrose for preparation of the parenchymal cell-enriched homogenate, by the lower speed-nuclear pellet loaded on the zonal centrifuge and by a modified gradient form. A higher yield in plasma membrane protein as well as a higher purification according to 5′-nucleotidase was obtained by this modified procedure. In additon, our data indicate a plasma membrane preparation free of other cell organelles. The use of isoosmotic sucrose through all the course of the present isolation technique permits better preservation of other cellular components such as mitochondria and nuclei. It is possible that the method for the isolation of parenchymal cells remove surface components of which

we are unaware thus providing the high specific activity of the plasma membrane marker enzymes.

ACKNOWLEDGEMENTS

This work was supported by grants from The National Cancer Institute of Canada, and also by grants from La Fondation J. H. Biermans and Les Fondations J. Rhéaume. D. G. is a Fellow of the Medical Research Council of Canada, and G. de L. and S. K. are Research Associates of The National Cancer Institute of Canada.

We are grateful to Miss Andrée Lemyre for excellent technical assistance.

REFERENCES

1 Neville, D. M. (1960) J. Biophys. Biochem. Cytol. 8, 413–422
2 Emmelot, P. and Bos, C. J. (1962) Biochim. Biophys. Acta 58, 374–375
3 Emmelot, P. and Bos, C. J. (1966) Biochim. Biophys. Acta 121, 375–385
4 Emmelot, P., Bos, C. J., Benedetti, E. L. and Rumke, P. H. (1964) Biochim. Biophys. Acta 90, 126–145
5 Lansing, A. I., Belkhode, M. L., Lynch, W. E. and Lieberman, I. (1967) J. Biol. Chem. 242, 1772–1775
6 Lieberman, I., Lansing, A. I. and Lynch, W. E. (1967) J. Biol. Chem. 242, 736–739
7 Lieberman, I., Ray, T. K. and Lansing, A. I. (1968) in Biological Properties of the Mammalian Surface Membrane (Manson, L. A., ed.), pp. 33–45, Wistar Institute Press, Philadelphia
8 Herzenberg, L. A. and Herzenberg, A. L. (1962) Proc. Natl. Acad. Sci. U.S. 47, 762–771
9 Takeuchi, M. and Terayama, H. (1965) Exp. Cell Res. 40, 32–44
10 Coleman, R., Michell, R. H., Finean, J. B. and Hawthorne, J. N. (1967) Biochim. Biophys. Acta 135, 573–579
11 Marinetti, G. V. and Gray, G. M. (1967) Biochim. Biophys. Acta 135, 580–590
12 Graham, J. M., Higgins, J. A. and Green, C. (1968) Biochim. Biophys. Acta 150, 303–305
13 Goldfisher, S., Essner, E. and Novikoff, A. B. (1964) J. Histochem. Cytochem. 12, 72–95
14 El-Aaser, A. A. and Reid, E. (1969) Histochem. J. 1, 439–457
15 Hanks, J. H. and Wallace, R. E. (1949) Proc. Soc. Exp. Biol. Med. 71, 196–200
16 De Lamirande, G. (1964) Cancer Res. 24, 742–750
17 Hinton, R. H., Burge, M. and Hartman, G. C. (1969) Anal. Biochem. 29, 248–256
18 Touster, O., Aronson, N. A., Dulaney, J. T. and Hendrickson, H. (1970) J. Cell Biol. 47, 604–618
19 Michell, R. H. and Hawthorne, J. N. (1965) Biochem. Biophys. Res. Commun. 21, 333–338
20 El Aaser, A. A. and Reid, E. (1969) Histochem. J. 1, 417–439
21 Swanson, M. A. (1955) Methods in Enzymol. 2, 541–543
22 Gianetto, R. and De Duve, C. (1955) Biochem. J. 59, 433–435
23 Weaver, R. A. and Boyle, W. (1969) Biochim. Biophys. Acta 173, 377–388
24 De Lamirande, G., Morais, R. and Blackstein, M. (1967) Arch. Biochem. Biophys. 118, 347–351
25 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
26 Munro, H. N. and Fleck, A. (1966) Analyst 91, 78–88
27 Giles, K. W. and Myers, A. (1965) Nature 206, 93
28 Erecinskà, M., Sierakowska, H. and Shugar, D. (1969) Eur. J. Biochem. 11, 465–471
29 Daoust, R. and Cantero, A. (1959) Cancer Res. 19, 757–762
30 Berman, H. M., Gram, W. and Spirtes, M. A. (1969) Biochim. Biophys. Acta 183, 10–18
31 Stein, Y., Widnell, C. and Stein, O. (1968) J. Cell Biol. 39, 185–192
32 Wattiaux-De Coninck, S. and Wattiaux, R. (1969) Biochim. Biophys. Acta 183, 118–128
33 Hinton, R. H., Fitzsimons, J. T. R., Dobrota, M. and Reid, E. (1970) Eur. J. Biochem. 12, 349–361
34 Emmelot, P. and Bos, C. J. (1972) J. Membrane Biol. 9, 83–104