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ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANES FROM KREBS II ASCITE CELLS USING PERCOLL GRADIENT

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1. Plasma membranes were isolated from Krebs II ascite cells grown in the mouse. Cells were disrupted by nitrogen cavitation in an isotonic alkaline buffer containing magnesium and ATP. Isolation was performed in an alkaline-buffered self-generating gradient of Percoll with an angular rotor. At each step of the preparation, the pH appeared as the critical aspect of our procedure. 2. External membrane markers were concanavalin A and 5′-nucleotidase (EC 3.1.3.5). They reached a relative specific activity of 10, whereas this value was only of 0.7 for the endoplasmic reticulum marker, NADH dehydrogenase (EC 1.6.99.3). 3. Plasma membrane from 4 ml packed cells were isolated within 1 h after homogenization with good yield: 50% and 67% of total [^{3}H]concanavalin A and 5′-nucleotidase, respectively, were recovered in the two plasma membrane fractions. 4. Electron microscopy examination showed the presence of vesicles of different sizes devoid of other structural contaminants. 5. Using the specific binding of concanavalin A to the external cell membrane, it was calculated that about 50% of the total cell phospholipid and 10% protein are located in the plasma membrane. Their sphingomyelin content is much higher than in the whole cell, in contrast to phosphatidylinositol, known as a more specific endoplasmic reticulum phospholipid.

Introduction

Ascite cells provide a convenient tool for investigation on plasma membranes from a neoplastic tissue. Numerous methods are now available for their isolation [1]. Regarding only Ehrlich ascite cells, plasma membranes have been isolated on sucrose gradient [2], by phase partition between aqueous polymer solutions or after cell stabilization with a chemical reagent [3]. But the procedures developed so far for ascite cells appear lengthy, involving differential centrifugations followed sometimes by very long equilibrium centrifugation. Moreover, the plasma membrane yield is generally low, so that it is not known whether the material isolated is representative of the total external cell membrane. In recent times, new approaches have been developed to obtain the intact cell envelope. The general idea has been to stick the cells on several types of solid support and then to remove the intracellular content by hypotonic shock [4] or by mechanical disruption [5]. However, removing the membranes from their support leads to very dispersed material, which could be a disadvantage for large-scale preparations. Proceeding from the same methodology, lectins have been used by the way of immunoprecipitation or linked to Sepharose [6]. Nevertheless, there exist discrepancies as to the types of membrane vesicle isolated [7].

Considering only two parameters, i.e., rapidity and plasma membrane yield, it appeared that no suitable purification process was available, at least for ascite cells. As the heaviest step is generally the

gradient, the use of self-generating gradients constituted by colloidal silica particles as Percoll® (Pharmacia) has been an improvement [8]. The product is not cytotoxic and has no osmotic effect that could shrink the subcellular particles, which sediment to their own density. Thus, plasma membranes have been isolated from several normal cells, including liver cells, epithelial cells and human platelets [9–11].

Here we report a convenient method for plasma membrane isolation from Krebs II ascite cells. Cell disruption conditions have been particularly thoroughly analysed. Differential centrifugations have been reduced, so that a low-speed supernatant obtained from the homogenate is quickly mixed with Percoll. Our new procedure is about 10-times more rapid than that previously described [12]. Its efficiency is proved by the enrichment in plasma membrane markers, their high content of sphingomyelin and cholesterol, and electron microscopy examination.

Material and Methods

Cell isolation

Ascitic fluid was harvested from 6–8 female Swiss mice which had been inoculated 7–9 days before. The strain was maintained by intraperitoneal injection of 0.3 ml ascitic fluid, i.e., $20 \cdot 10^6$ cells per mouse, in the presence of antibiotics.

Cell washings were performed at room temperature. Ascitic fluid was immediately diluted 4-fold with a calcium-free Tyrode buffer to avoid coagulation. Cells were spun down at $200 \times g$ for 5 min. The pellet was then washed by 40–50-times its volume of the previous buffer. Finally, packed cells were washed by the labelling buffer, i.e., 100 mM KCl/5 mM $MgCl_2$/25 mM Tris-HCl (pH 7.4) and suspended in the same medium to about a 30% (v/v) cell suspension.

Cell labelling by tritiated concanavalin A

20 ml of the 30% cell suspension were incubated with 2 μCi (equivalent to 0.3–0.7 $\mu g/10^7$ cells) of [^{3}H]concanavalin A (Amersham), for 15 min at room temperature under gentle magnetic stirring. Cells were spun down and the supernatant was discarded. The pellet was washed twice by 40-times its volume with the labelling buffer, followed by a washing with the isotonic lysis buffer constituted by 100 mM KCl/5 mM $MgCl_2$/1 mM ATP/25 mM Tris-HCl (pH 9.6). Final cell suspension was 10% (v/v) in the lysis buffer. Approx. 70% of the [^{3}H]concanavalin A remained fixed on the cells.

Plasma membrane isolation

All operations were carried out at 4°C.

Cell disruption. Lysis was realized by the nitrogen cavitation procedure [13]. Cell suspension (10%, v/v) was equilibrated at 4°C for 20 min with 60 atm. of N_2 in a Kontes pressure homogenizer (Kontes, Vineland, NJ, U.S.A.) and then released dropwise. Aliquots of the homogenate were kept for enzyme, radioactivity and total lipid phosphorus measurements. A part of the homogenate was centrifuged at $200000 \times g$ for 30 min to measure the amount of unbound concanavalin A.

Plasma membrane separation. The homogenate was centrifuged at $1000 \times g$ for 10 min to sediment nuclei and rare cellular debris. The pH of the supernatant was adjusted again to 9.6 with a few drops of 0.25 M NaOH. 4 ml were added to a previously prepared mixture of 11 ml Percoll/2.2 ml distilled water, buffered with 4.8 ml 400 mM KCl/20 mM $MgCl_2$/100 mM Tris-HCl (pH 9.6), so that final conditions were isotonic. Subcellular organites and membranes were then separated by centrifugation at 40000 rpm for 10 min at the plateau, in a fixed angle rotor (Beckman rotor 60 Ti). The speed was decreased with the brake, which was switched off at 1000 rpm. Fractions of 2 ml were harvested from the top of the transparent thick-wall test tube and the pH was immediately adjusted to 7.4 by adding 1 ml of a buffer containing 100 mM KCl/5 mM $MgCl_2$/50 mM Tris-HCl (pH 7.4).

Radioactivity and spectrophotometric enzyme determinations were first carried out. The fractions with the highest amount of [^{3}H]concanavalin A were diluted 3-fold with the labelling buffer and centrifuged at $200000 \times g$ for 45 min. Membranes remained stuck at the surface of the Percoll cushion. After collecting them in the labelling buffer, a few seconds of sonication were necessary in some cases to obtain homogeneous material suspension.

Enzymatic determinations

Membrane markers. The 5'-nucleotidase (EC 3.1.3.5) procedure was adapted from Avruch and Wallach [14] and from Johnson and Robinson [15]. The activity was assayed in 1 ml incubation medium containing 2 mM AMP with 10 nCi [^{3}H]AMP per sample, 0.2 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.0), (the optimum pH in our case) and 100 μl of gradient fractions. Incubation at 37°C was continued for 60 min, and the reaction was stopped with 0.3 ml of 0.2 M zinc sulphate. For an effective precipitation of proteins and unhydrolysed AMP, a waiting period of 30 min at +4°C was necessary before adding 1 ml of freshly prepared 0.2 M barium hydroxide. Under these conditions, we obtained the lowest blank values, i.e., 0.3% of the initial radioactivity.

The NADH dehydrogenase (EC 1.6.99.3) [14,16], also called NADH diaphorase [2,12], was measured with minor modifications of the initial technique of Wallach and Kamat [13]. The best conditions were with equal concentrations (0.7 mM) of NADH and ferricyanide in a 100 mM Tris-HCl (pH 7.4) buffer. Proteins (100 μg or 100 μl of gradient fractions) were added to 1 ml incubation mixture. The enzymatic reaction was determined by ferricyanide reduction at 410 nm instead of NADH oxidation because of the quenching effect from Percoll.

CDP choline : 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) was determined according to Coleman and Bell [17] with 50 μl gradient fraction as enzyme source. The activity of NADH dehydrogenase (EC 1.6.99.3) was controlled in the presence of cytochrome *c* as a receptor by the method of Pommier et al. [18] without antimycin A.

Subcellular organite markers. Lysosomes were detected by measuring the *N*-acetyl-β-D-glucosaminidase as described by Day et al. [19]. Succinate dehydrogenase (EC 1.3.99.1) for mitochondria was assayed by the method of King [20] in the presence of 0.1% (w/v) sodium deoxycholate.

Analytical methods

Radioactivity was evaluated with a Kontron Intertechnique liquid scintillation spectrometer (Type SL 4000) with automatic quenching correction using Picofluor (Packard, U.S.A.) as scintillation fluid. Proteins were determined by the method of Lowry et al. [21] in the presence of sodium dodecyl sulphate (0.07%, w/v). Bovine serum albumin was used as standard. Phospholipids were extracted according to Bligh and Dyer [22]. The separation was performed by the bidimensional system of Esko and Raetz [23] on 0.25 mm thick silicagel G plates (Merck). Phosphorus was estimated by a modification [24] of the method of Fiske and Subbarow. The cholesterol determination method was from Liebermann [25].

Electron microscopy

Plasma membranes were pelleted at 200000 × *g* for 45 min and then fixed with 2% glutaraldehyde in 0.05 M Sorensen's phosphate buffer (pH 6.8) for 60–90 min. Post-fixation was realized in the same buffer with 1% osmium tetroxide for 2 h, followed by alcohol dehydratation and embedding in epon. After polymerization, ultrathin sections were stained with uranyl acetate and lead citrate, and examined in Hitachi H 300 electron microscope.

Results

Fractionation of Krebs II cells

In the procedure described herein, plasma membranes were isolated from a low-speed supernatant containing about 90% of both membrane and organite markers (Table I). Such release of structures from the 1000 × *g* pellet was obtained by including ATP and magnesium in the buffer and by breaking the cells at an alkaline pH, with a cell suspension of 10% (v/v). Higher cell concentration (30%, v/v) and lower lysis pH (7.4) led to a completely opposite pattern, since 80% of [^{3}H]concanavalin A were recovered in the 1000 × *g* pellet. When this pellet was fractionated on Percoll gradient, a large part of plasma membranes remained stuck to nuclei. For this reason, conditions were checked to avoid the sedimentation of membranes prior to the gradient step.

Separation of plasma membranes on Percoll gradient

Alkaline pH was maintained for gradient preparation, since macroscopic observations have shown strong clump formation at pH 7.4, which

TABLE I

MARKER DISTRIBUTION IN THE HOMOGENATE

Results are mean of three experiments and are percentages of total contents. The solubilized and particulate amounts in the 1000×*g* supernatant were determined after a centrifugation at 200000×*g* for 30 min. Values in parenthesis indicate the part of structure-bound markers when both particulate amounts of the 1000×*g* pellet and supernatant are taken as 100%.

Marker	Pellet 1000×*g*	Supernatant, 1000×*g*	
		Particulate	Solubilized
[^{3}H]Concanavalin A	9.3	69.7 (88.2)	21.0
5′-Nucleotidase	9.2	78.0 (89.4)	12.8
NADH dehydrogenase	7.7	74.9 (90.6)	17.4
N-Acetyl-β-D-glucosaminidase	5.1	68.7 (93.1)	26.2
Succinate dehydrogenase	8.6	76.3 (90.0)	15.1

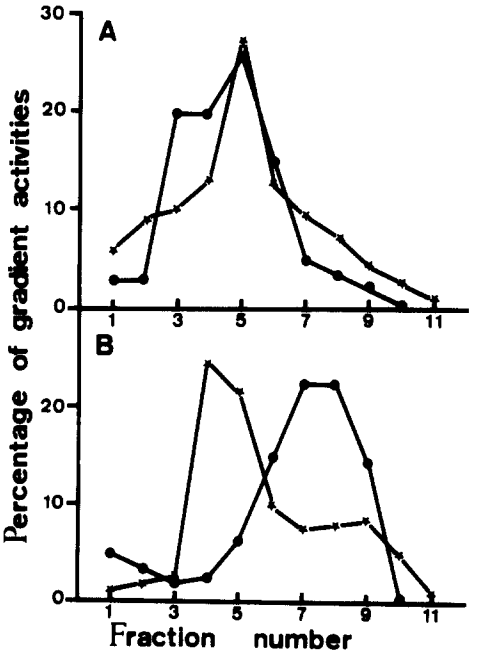


Fig. 1. Effect of pH on the separation between endoplasmic reticulum and plasma membranes. In part A, [^{3}H]concanavalin A (★) and NADH dehydrogenase (●) were focused in a single peak when cell lysis and pH were performed at pH 8.6. At pH 9.6, each type of membrane was perfectly separated (part B).

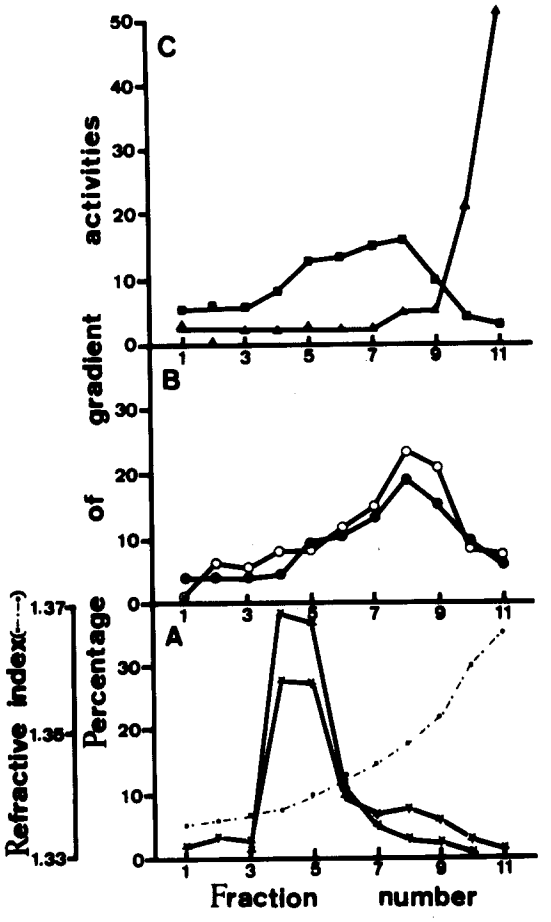
decreased at higher pH. This effect is illustrated in Fig. 1, which demonstrated that a pH of 9.6 was needed to obtain an effective separation between endoplasmic reticulum and plasma membranes. Values ranging between 8.6 and 9.6 were not so efficient and gave intermediate patterns.

The results of a typical plasma membrane fractionation are represented in Fig. 2. Part A shows that 5′-nucleotidase activity displayed exactly the same profile as [^{3}H]concanavalin A. The enzyme activity was determined using substrate labelled on the adenosine moiety to avoid interference with phosphate released by an ATPase, as suggested by previous experiments. In part B, the same behaviour was found for NADH dehydrogenase and diacylglycerol cholinephosphotransferase. Thus, the latter enzyme appears as a convenient marker for endoplasmic reticulum. Identical results were obtained with NADH dehydrogenase with cytochrome *c* as a receptor (not shown). As for other subcellular organites, mitochondria were precipitated at the bottom of the gradient, whereas lysosomes banded at a position similar to endoplasmic reticulum (Fig. 2C). Localization of lysosomes appeared to be very sensitive to a correct pH adjustment of the gradient load.

The yield of the purification procedure was estimated using results of Table I and Fig. 2. Based on the structure-bound amount of markers contained in low speed supernatant, the final recovery of plasma membranes ranged between 50% (concanavalin A) and 67% (5′-nucleotidase), as compared to total homogenate. Discrepancies between the two markers resulted from their distinct solubilized amount introduced on the gradient: concanavalin A was twice more solubilized than 5′-nucleotidase (Table I). Actually, final enrichments of plasma membrane markers are very close in fractions 4 and 5 (Table II).

Electron microscopy

Electron microscope examination showed a distribution of mostly membrane vesicles (Fig. 3). In fraction 4 (Fig. 3A), two kinds of vesicle of differ-



ent size could be observed. Some of them contained particles of different electron density, which suggests trapping of hyaloplasmic material. Membranes of dictyosomes could not be identified. Fraction 5 (Fig. 3B) revealed the presence of vesicles with larger size than in fraction 4 containing hyaloplasmic material associated with lipoprotein structures. In contrast to with fraction 4, membrane sheets were more numerous and very electron-dense granulations were bound to membranes. Based on several observations, we assume that they are remaining particles of Percoll, their small size being consistent with that of Percoll beads (about 17 nm, see Ref. 8). Similar granulations can be detected in other preparations using this product [10]. The general aspect of the picture from fraction 4 is very close to that of 'light' membranes from Ehrlich-Lettré cells, whereas Fig. 3B resembles the 'heavy' membranes [26]. Another

Fig. 2. A typical plasma membrane separation from the low-speed supernatant. Conditions were those described in Material and Methods. Percoll content of gradients was 50%, v/v. Part A. Plasma membrane markers; [^{3}H]concanavalin A (★) and 5'-nucleotidase (☆). Part B. Endoplasmic reticulum markers; NADH dehydrogenase (●) and cholinephosphotransferase (○). Part C. *N*-Acetyl-β-D-glucosaminidase for lysosomes (■) and succinate dehydrogenase for mitochondria (▲).

TABLE II

RELATIVE ACTIVITIES OF ENZYME AND [^{3}H]CONCANAVALIN A IN DIFFERENT FRACTIONS OF PERCOLL GRADIENT

Specific activities are expressed in dpm/mg protein (a), nmol/min per mg protein (b) and nmol/h per mg protein (c). Values in parenthesis are relative specific activities of fractions to homogenate (enrichment factor). Results are given as means ± S.D. from three experiments. n.d., not determined.

Marker	Homogenate	Gradient fractions		
		4	5	6
[^{3}H]Concanavalin A [a]	4270±90	45743±10637 (10.7)	39225±1500 (9.2)	n.d.
5'-Nucleotidase [c]	164±39	1698±362 (10.3)	972±215 (5.9)	419±135 (2.5)
NADH dehydrogenase [b]	175±15	123±21 (0.7)	200±21 (1.1)	309±89 (1.8)
N-Acetyl-β-D-glucosaminidase [c]	560±60	878±188 (1.6)	1999±420 (3.6)	2230±470 (4.0)
Succinate dehydrogenase [b]	61000	- [d]	- [d]	- [d]

[d] Not detected.

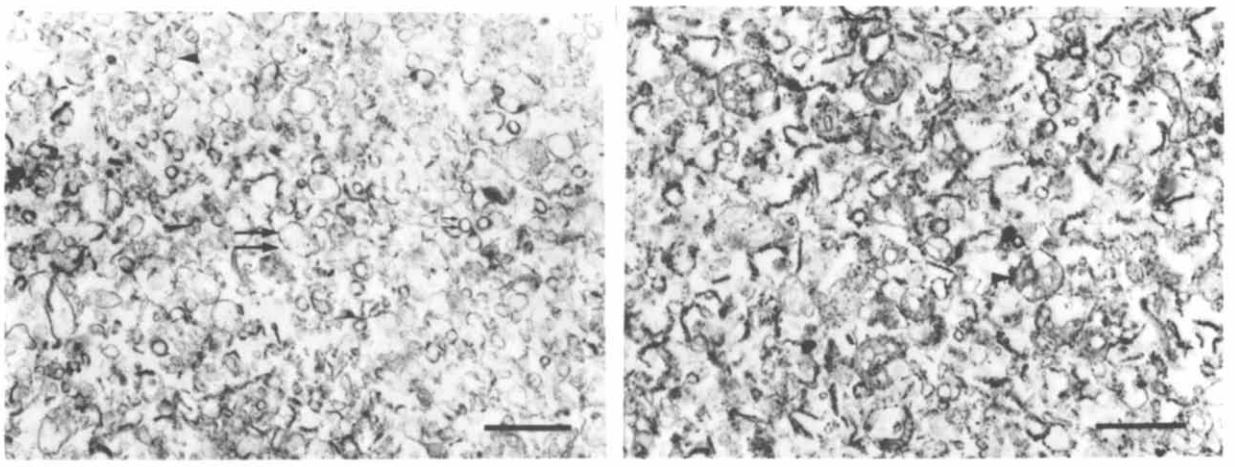


Fig. 3. Electron micrographs of a thin-sectioned preparation of plasma membranes. A (Left). Material from fraction 4 is constituted by concentric packed (◄), large (→) and small (→) vesicles. (Magnification, ×11330). B (Right). Fraction 5 contains very large vesicles (►) with numerous smaller ones and membrane sheets. (Magnification, ×11330: the bar represents 1 μm in each case.)

similitude is the distinct color of our membrane pellets; white in the case of fraction 4 and light yellow for fraction 5.

Protein and phospholipid content of isolated plasma membranes

As already used by Perret et al. [11], the specific binding of concanavalin A to the external cell surface allowed us to calculate the protein and phospholipid content of the plasma membrane compared to that of the whole cell (Table III). In Krebs II cells, about 10% of the total proteins and 50% of the total phospholipids are thus located in the plasma membrane. This latter result suggests that one half of the total cell membranes is constituted by the external cell membrane.

Phospholipid compositions are summarized in Table IV. In plasma membranes two phospholi-

TABLE III

ESTIMATION OF THE PHOSPHOLIPID AND PROTEIN CONTENT OF PLASMA MEMBRANES

Results (mean ±S.D. from three experiments in duplicate) represent the specific radioactivity of [^{3}H]concanavalin A expressed in dpm/mg protein (column 1) or in dpm/μmol phospholipid (column 2). Since the results reported above showed that particulate concanavalin A in the cell was coming only from plasma membrane, the ratio A/B (A = particulate concanavalin A/total cell proteins or phospholipids, B = plasma-membrane concanavalin A/plasma membrane proteins or phospholipids) gives the proportion of the total cell proteins and phospholipids in plasma membranes (see Ref. 11).

	Proteins (1)	Phospholipids (2)
Specific radioactivity		
(A) Total cell	3373 ± 71	35428 ± 2060
(B) Plasma membrane	37872 ± 2308	64276 ± 3295
A/B	0.089	0.551

TABLE IV

PHOSPHOLIPID COMPOSITION AND CHOLESTEROL CONTENT OF ISOLATED PLASMA MEMBRANES

Results are given as mean ±S.D. from three experiments in duplicate and are expressed in molar percent of total.

	Total cell	Plasma membranes
Sphingomyelin	13.0 ±0.8	25.8 ±1.2
Phosphatidylserine	6.0 ±0.4	9.8 ±1.1
Phosphatidylinositol	7.1 ±0.4	3.5 ±0.3
Phosphatidylethanolamine	23.6 ±1.1	28.1 ±0.9
Phosphatidylcholine	47.3 ±1.0	30.3 ±0.9
Lysophosphatidylcholine	0.5 ±0.1	traces
Cardiolipin	2.5 ±0.4	2.5 ±0.4
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$ (molar)	0.34 ± 0.02	0.62 ± 0.04

pids were increased in comparison to the total cell: phosphatidylserine and sphingomyelin (about twice). In contrast, phosphatidylinositol, known as a more specific endoplasmic reticulum phospholipid, was decreased to the same extent. Despite these relative variations, the sum of the three major phospholipids (sphingomyelin, phosphatidylcholine, phosphatidylethanolamine) was similar in plasma membranes and in the cell (84.2 and 83.9%, respectively), and the amount of cardiolipin (2.5%) did not change. On the other hand, the cholesterol/phospholipid ratio was twice as high in the plasma membrane.

Discussion

In this paper, we report a new isolation procedure of plasma membrane from ascite cells. The starting material is a pure population of Krebs II cells since, as shown by cell numeration, the centrifugation speed used for washing eliminates red blood cells and macrophages does not exceed 2%. Rapidity and efficiency appear to be the main advantages of this procedure. These are a consequence of the use of Percoll as self-generating gradient and of the suppression of differential centrifugations.

We assume that a high yield of plasma membranes is required to avoid their sticking to other subcellular organites. This has been achieved by using an appropriate lysis buffer. Based on preliminary observations and on literature data, magnesium [27,28] and ATP [29] were included in this lysis buffer in order to prevent gel formation of actin filaments [30]. In our case, lower concentrations of ATP than those generally used [11] were still effective and both effects of magnesium and ATP were evidently enhanced by alkaline pH. High pH values induce a negatively charged buffer that might prevent subcellular particle clumping. Since 10% only of total plasma membranes remain in the nucleus pellet (Table I), the sedimentation of a part of them in this fraction, as generally encountered in the literature, is not specific to a class of membranes as suggested by the results of Touster et al. [31]. The step of the $1000 \times g$ centrifugation cannot be suppressed in so far as the rare clumps thus precipitated would be uniformly distributed in the gradient.

The selective shift of endoplasmic reticulum to higher density comparatively to plasma membrane on the gradient appears as a consequence of the mechanism elaborated by Steck et al. [32] where pH and ionic strength selectively modify the internal volume of membrane vesicles according to their origin, with a subsequent effect on their gradient migration. This mechanism necessitates gradients with a low osmotic activity, as in the case of Percoll.

The unusual pH used in this report must be discussed with regard to eventual damage to cells and structures. It should be borne in mind that our pH is not more distant from the neutrality than the value of 5.0 used in some procedures [4,5,33] and that Percoll is not altered, since it can be buffered from pH 5 to 10. Krebs cells are allowed to remain only 30 min at pH 9.6, whereas it was shown that maximal injury of macrophages occurred after an overnight incubation at pH 10.6 [34]. As monitored by the solubilized amounts of markers (Table I), our procedure is not specially drastic [12]. Nuclei are rather large in the case of ascite cells and their membrane is a potential contaminant; but these organites are eliminated within the 10 min following cell disruption. Their pellet was not gelatinous, which indicates that the nuclei were sedimented intact [35]. It is noteworthy that nucleus membrane [28,36,37] as well as mitochondria external membrane [33] can be characterized by the NADH dehydrogenase with cytochrome *c* as a receptor. As the latter displayed on the gradient the same profile as other endoplasmic reticulum markers, such a contaminant can thus be excluded. Some structural effects of alkaline pH have been observed by Steck [38] on erythrocyte membranes, but after preparation in hypotonic medium without cations. On the other hand, an advantage of alkaline pH is to inhibit lysosomal acid hydrolases [39], making protease inhibitors useless [10].

Electron microscopy shows vesicles similar to other preparations [10,26]. The light morphological differences between fractions 4 and 5 certainly refer to plasma membrane mosaicism [7], which could be a consequence of temperature-induced phase separation subsequent to cell cooling for homogenization [28]. Thus, both fractions were analysed as a pool.

As for their purity, there is a good homogeneity between [^{3}H]concanavalin A and 5′-nucleotidase enrichments (Table II). The utilization of a chemical probe avoids enrichment variations as observed with enzymatic markers [28] owing to partial enzyme inactivation or sideness with non-permeant substrates. As previously observed on platelets [11], we found no redistribution of the lectin. This is of importance in so far as internal cell membranes are potential binders [40] and that endocytosis could internalize concanavalin A [41]. But the concentrations used are very far from those required for cell agglutination [42] or for a physiological effect such as mitogenic induction [43], so that 5′-nucleotidase was not inhibited, the enzyme being a lectin receptor [44]. By using concanavalin A, we could calculate the proportion of plasma membrane protein and phospholipid in the cell. The latter value is very close to that found by Perret et al. [11], using the same methodology, in a normal cell. The plasma membrane protein content is much higher than those generally reported [2,16,28] but the phospholipid-to-protein ratios are in the same range of magnitude as in other reports [2,16,31,33] (Table III).

Lipid analysis also shows a cholesterol/phospholipid ratio similar to literature data [15,35,45]. Phospholipid analysis reveals a rather high content of phosphatidylserine as compared to some neoplastic cell plasma membranes [24,46,47] and similar to that of platelet [11]. On the other hand, the high enrichment in sphingomyelin provides additional support for the purity of isolated membranes [48], as well as the increase in cholesterol/phospholipid ratio. Significant amounts of cardiolipin were found in the purified plasma membranes which were not contaminated by mitochondria, on the basis of enzyme markers. This finding gives further support to the abnormal presence of cardiolipin in plasma membrane of neoplastic cells [49–51].

Concluding remarks

The main characteristic of our procedure is to avoid important losses of plasma membranes, since 90% of them mixed with Percoll were focused in two fractions of gradient. The total time to get a washed membrane pellet is remarkably the same as that reported by Hourani et al. [52] (two-phase polymer separation) and Cohen et al. [33] (bead binding). Thus the procedure reported herein is among the most rapid and convenient of the methods reported so far.

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