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Isolation of the plasma membrane and organelles from Chinese hamster ovary cells

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Two methods are described enabling the plasma membrane from Chinese hamster ovary (CHO) cells to be obtained rapidly, relatively pure and with a good yield. In both cases, cells were disrupted by nitrogen cavitation in an isoosmotic buffer either at pH 5.4 or at pH 7.4. In the first approach, cells were lysed at pH 7.4 and the plasma membrane and cell organelles were isolated on a self-generated gradient of Percoll, at neutral pH. Mitochondria and endoplasmic reticulum were recovered in the denser fractions, plasma membrane fragments were found in the lighter fractions, but always contaminated by lysosomes. Because lysosomes were found to sediment in acidic conditions, cells were lysed at pH 5.4 and presedimentation ($1500 \times g$) of the cell homogenate at the same pH enabled more than 80% of the lysosomes to be removed. Then, ultracentrifugation of the supernatant over a Percoll gradient at neutral pH yielded plasma membrane fractions practically free of lysosomes with an enrichment ratio of 3 and fractions of mitochondria and endoplasmic reticulum with enrichment ratios of 17 and 6, respectively. A major problem was encountered in the final step of elimination of Percoll from the purified plasma membrane fractions. Whatever the technique used for eliminating Percoll, plasma membranes were observed to be contaminated by a Percoll constituent which prevented further purification and biochemical identification of the lipids extracted from these membrane fractions to be carried out. A second method of plasma membrane preparation was tested consisting first in the coating of the cell surface with positive colloidal silica which was stabilized by an anionic polymer. Then, and through differential centrifugations, plasma membrane fractions were easily obtained within less than 1 h, with a yield of 65% and an enrichment ratio of 7. The coating pellicle was quantitatively removed thus enabling any biochemical manipulation of the plasma membrane to be carried out. The lipids present in the plasma membrane of CHO cells were analyzed and are described, both in terms of headgroup and acyl chain composition.

Introduction

Recently, we have developed a new approach involving anthracene as a fluorescent and photoactivatable group to investigate both the lateral distribution and the dynamics of lipids in biological membranes. This hydrophobic chromophore, which is well suited for labeling the hydrophobic core of the membrane, was introduced into lipids in the form of 9-(2-anthryl)nonanoic acid, whose synthesis and physical chemical properties have already been described [1,2].

Taking advantage of the fluorescence and photodimerization properties of anthracene, we have shown that after incorporation into membrane lipids, this chromophore can be used to study the lateral diffusion rate of the labeled molecules using fluorescence recovery after photobleaching (FRAP) techniques [3,4] and to study the lateral distribution of lipids in membranes after photo-cross-linking of adjacent anthracene-labeled molecules and subsequent identification of the photodimers [5,6]. Combined with metabolic incorporation of the anthracene-fatty acid into membrane lipids [7,8], these new approaches were successfully applied to the plasma membranes of eukaryotic (Chinese hamster ovary cells) [8] and prokaryotic (*Micrococcus luteus*) cells [9]. As a main result, the photo-cross-linking technique provided strong evidence for the existence of large heterogeneity in the lateral distribution of phosphatidylglycerol and dimannosyldiacylglycerol (the two

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Abbreviations: FRAP, fluorescence recovery after photobleaching; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography; LDH, lactate dehydrogenase; ConA, concanavalin A; EGTA, ethyleneglycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; Mes, 2-(*N*-morpholino)ethane sulfonic acid.

major lipids) in the bacterial membrane [6,10], while FRAP experiments clearly indicated the occurrence of lipid microdomains in the plasmalemma of CHO cells [9].

To go further in the elucidation of the composition and organization of these lipid microdomains in the CHO plasma membranes, and in particular by using the photo-cross-linking approach, one prerequisite is to have purified plasma membranes. These membranes should be obtained pure, with a good yield and as rapidly as possible, and in a way such that the lipids might be extracted free of any external contaminant.

CHO cells provide an interesting model system which is widely used for many investigations in cellular biology. The possibility of easily obtaining well purified plasma membranes and organelles might be of great interest for many laboratories. Surprisingly, only a few reports deal with the purification of the plasma membrane of CHO cells. They do not meet the above requirements of purity, yield and rapidity of isolation.

In the present work, two methods of membrane preparation were assayed. One, developed by Perret and co-workers [11–16], which uses a Percoll gradient. After adaptation of the method to CHO cells, plasma membranes and organelles were obtained relatively pure but difficulties were encountered in the last step of extraction of the lipids from the plasma membranes. The lipids were systematically contaminated by a Percoll constituent which proved to be very difficult to remove. The second procedure tested was that described by Chaney and Jacobson [17], which consists in coating intact cells with a pellicle of positively charged colloidal silica particles which is then stabilized by an anionic polymer. After disruption of the cells, the plasma membranes which now exhibit an enhanced density are easily isolated by low speed differential centrifugations. This technique has been adapted to the preparation of CHO plasma membranes and their lipid components have been characterized biochemically.

Materials and Methods

Chemicals. Salts and solvents were of analytical grade.

Cell culture. CHO cells were grown in suspension at 37°C under gentle agitation in Eagle's minimal essential medium [18] (MEM 0111, Eurobio, France) supplemented with vitamins, amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml) and 8% fetal calf serum (Boehringer, Germany). Cell density was maintained between $3 \cdot 10^5$ and $6 \cdot 10^5$ cells/ml by daily dilution of the cell suspension in the same growth medium, in order to keep the cells in the exponential phase of growth. Cell density was determined by a direct counting of the cells in the microscope.

Labeling of the cell surface. This step is common to both types of preparation of plasma membranes we have tested. CHO cells, in suspension in the culture medium, were centrifuged and the pellet was washed twice with phosphate-buffered saline (PBS: 0.15 M NaCl, 0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, pH 7.4) by centrifugation. Cell pellet was resuspended at a density of 10^7 cells/ml in PBS and incubated for 20 min at 20°C with [^{3}H]concanavalin A (^{3}H-ConA, 1 μCi/ml, Amersham, UK). The cells were centrifuged ($400 \times g$, 5 min) and washed twice with PBS. In these conditions, all the non-specific adsorbed lectin was removed from the cell surface. After this surface labeling, all steps were performed at 4°C.

Preparation of plasma membrane using Percoll gradients. Cells were suspended in the lysis buffer (40 mM Tris, 90 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 1.5 mM EGTA, 1 mM PMSF, pH 7.4 or 5.4) at a density of $3 \cdot 10^6$ cells/ml and equilibrated at 4°C for 10 min with nitrogen at a pressure of 10 atm in a Kontes pressure homogenizer (Kontes, USA) [19].

The cell suspension was released dropwise from the homogenizer and then centrifuged at $1500 \times g$ during 10 min to remove intact cells and nuclei. The cell pellet was recovered and tested for its enzymatic and radioactive content. The supernatant was collected and mixed with Percoll (Pharmacia, Sweden) in the following proportions: 5 ml homogenate, 11 ml Percoll, 5 ml lysis buffer concentrated three fold. pH was adjusted to 7.4 with 0.5 M Na_2CO_3 solution. The mixture was centrifuged in a Beckman centrifuge using 60 Ti rotor at $70000 \times g$ for 15 min. Fractions of 2 ml were harvested from the top of the tube and tested for their radioactivity and various enzyme markers. To remove Percoll from the plasma membrane fractions, the fractions containing the highest amount of ^{3}H radioactivity were pooled, diluted 3-fold with PBS and centrifuged at $200000 \times g$ for 45 min. Plasma membranes were floating over the Percoll pellet. They were collected, diluted in PBS and centrifuged again at $200000 \times g$ for 45 min. Plasma membranes were collected as above. In order to precipitate residual Percoll, cold isopropanol with 2% acetic acid was added to this fraction. After centrifugation at $1500 \times g$ for 15 min, lipids were recovered in isopropanol and extracted according to the method of Bligh and Dyer [20] modified by Record et al. [21].

Preparation of plasma membrane using silica beads. As described in the Introduction, plasma membranes with enhanced density were obtained after fixation on the cell surface of positively charged colloidal silica beads stabilized by an anionic polymer.

Positively charged colloidal silica beads (30% suspension by weight) [17] were kindly provided by Drs. S. Jacobson and K. Auer (Massachussets, USA). Based on the results of these authors, polyacrylic acid (PM =

90000, Aldrich, USA) was selected as polyanion [17]. The formation of the silica-polyanion pellicle was performed as described by Jacobson et al. [17]. Cells were suspended in the attachment buffer (140 mM sorbitol, 20 mM Mes, pH 6.5) at a density of 10^7 cells/ml. For 2×10^7 cells, 2 ml of silica suspension at 8% by weight were added. After gentle mixing, 20 ml of wash buffer (70 mM NaCl, 20 mM Mes, pH 6.5) were added. The suspension was centrifuged at $400 \times g$ for 5 min in order to pellet the cells away from silica in excess. Cell pellet was suspended in 3 ml of wash buffer and then 3 ml of a solution of polyacrylic acid (2 mg/ml in wash buffer, pH 6.5) were added. After addition of 20 ml wash buffer, cell suspension was centrifuged in order to remove polyanion in excess. Cells were washed three times with 20 ml wash buffer.

Cell lysis was then carried out using a nitrogen cavitation procedure [19] in the following way. Cell pellet was resuspended in the lysis buffer (40 mM Tris, 90 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 1.5 mM EGTA, 1 mM PMSF, pH 5.4) at a density of $3 \cdot 10^6$ cells/ml and equilibrated at 4°C for 10 min with nitrogen at a given pressure (see Results) in a Kontes pressure homogenizer. Then, cell suspension was released dropwise from the homogenizer.

Lysate was fractionated by differential centrifugations. It was first centrifuged at $200 \times g$ during 3 min to remove intact cells. Plasma membranes were pelleted by centrifugation of the supernatant at $400 \times g$ and the centrifugation time was varied to optimize the recovery yield of plasma membranes (see Results). Cytosol, nuclei and intracellular organelles were found in the supernatant. Both fractions were tested for their enzymatic and radioactivity contents.

To remove the silica-polyanion pellicle from the plasma membrane sheets, pellets of coated plasma membranes were resuspended in 2 ml hypertonic PBS (0.2 M NaCl, 0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, pH 7.4). They were then sonicated three times for 3 min in a sonicating bath (80 kHz) and centrifuged at $400 \times g$ during 3 min. The silica pellet was resuspended in 2 ml of 200 mM PBS and submitted again to the same treatment. Using this procedure enabled all the silica-polyanion pellicle to be removed from the plasma membranes. Both supernatants, which contained plasma membranes, were collected and lipids were extracted according to Bligh and Dyer [20].

Enzymatic markers. The activity of *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), a marker of the lysosomes, was measured according to Day et al. [22]. Lysosomal β-glucuronidase (EC 3.2.1.31) was tested using Sigma kit. The NADH dehydrogenase (EC 1.6.99.3) also called NADH diaphorase, an enzyme of the endoplasmic reticulum, was measured according to a procedure adapted from Wallach and Kamat [19] and described by Record et al. [12]. The presence of mitochondria (succinate dehydrogenase, EC 1.3.99.1) was assayed according to the method of King [23]. The cytosolic lactate dehydrogenase (EC 1.1.1.27) was measured according to a procedure adapted from Wroblewski and La Due [24] and described by Chap et al. [25]. 100% lysis was estimated by addition of Triton X-100 at 0.1% in the assay.

Lipid analysis. Total lipid extracts from the plasma membranes prepared by both purification methods were chromatographied on Silica gel thin-layer plates (Merck, Germany) ($CHCl_3$/CH_3OH/CH_3COOH/H_2O, 65:25:20:4, v/v). Each phospholipid spot was scraped and analyzed for its phosphorus content according to Eaton and Dennis [26].

Relative percentages of phospholipids, cholesterol and triglycerides were determined using a COBAS FARA apparatus (Roche Diagnostic, France).

To analyze the fatty acid content of cell homogenates, of the total lipids extracted from plasma membrane fractions, of phosphatidylcholine or phosphatidylethanolamine purified by thin-layer chromatography from the plasma membrane, lipids were saponified (1 M KOH in CH_3OH/C_6H_6, 8:2, v/v) for 1 h at 60°C. Fatty acids were extracted with diethyl ether and converted into their methyl esters using diazomethane. Gas-liquid chromatography identification of esters was carried out with a Delsi apparatus equipped with a semi-capillary column (type OV1, 24 m long), over a temperature range from 100°C to 290°C.

Analytical methods. Proteins were dosed according to the method of Lowry et al. [27] using bovine serum albumin as standard. ^3H radioactivity was counted with a Packard liquid scintillation counter (model 1600 CA Tri Carb) using Lumasafe (Lumac, Belgium) as scintillation fluid.

Results

Preparation of plasma membranes over Percoll gradients

CHO cells in suspension were lysed at neutral pH after equilibration with nitrogen. A pressure of 10 atm led to 95% lysis. Lysate was then mixed with Percoll which, after centrifugation, generates a continuous gradient enabling membrane fragments differing in density to be separated. Fractions of 2 ml were collected from the top of the gradient and assayed for their radioactivity content and various enzymatic markers. Data presented in Fig. 1 shows that ^3H radioactivity associated with concanavalin A, the marker of plasma membranes, was found in the fractions of low density (fractions 2 and 3), as a single peak. This indicates that the conditions of incubation with ^3H-ConA were well adapted to CHO cells since no internalization of the lectin occurred. Lysosomes, revealed by *N*-acetyl-β-D-glucosaminidase, were found associated with the plasma

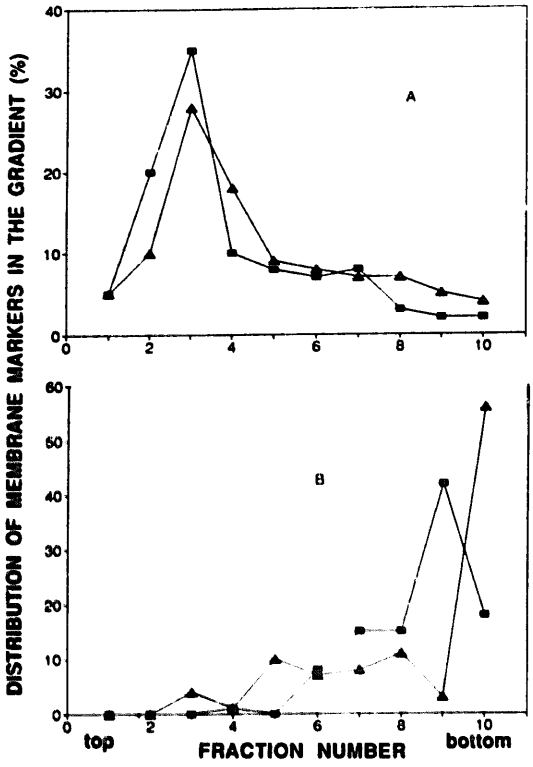

Fig. 1. Distribution of membrane markers after fractionation of plasma membrane and organelles of CHO cells on a self-generating Percoll gradient. pH lysis and pH gradient were 5.4 and 7.4, respectively. The data shown corresponds to one among seven experiments which gave similar distribution profiles. Marker activities are expressed as percentage of the total. Part A: ^{3}H-ConA (■) for plasma membranes, *N*-acetyl-β-D-glucosaminidase (▲) for lysosomes. Part B: NADH diaphorase (■) for endoplasmic reticulum, succinate dehydrogenase (▲) for mitochondria.

membrane fractions. Endoplasmic reticulum (NADH diaphorase) and mitochondria (succinate dehydrogenase) were well separated and mainly recovered in fractions 9 and 10, respectively.

In an attempt to decrease the rate of contamination of the plasma membranes by lysosomes, an effort was made to enhance the density either of the lysosomes, by addition of $FeCl_3$ [28], or of the plasma membranes, by addition of digitonin [29]. No better separation of these organelles was obtained.

It has been reported that the use of alkaline instead of neutral conditions for the Percoll gradient, improved the separation between plasma membranes and endoplasmic reticulum in Krebs II ascite cells [12] and in platelets [13]. In the case of CHO cells, varying the pH of the Percoll gradient from 5.4 up to 9.4 did not improve the separation of lysosomes and plasma membranes.

Changing the pH of the lysis step was also tested. Separation of the various organelles, in particular plasma membranes and lysosomes, on the Percoll gradient was not improved. In contrast, differences were observed in the sedimentation rate of the plasma membranes and lysosomes during the $1500 \times g$ centrifugation step of the homogenate, which preceded the gradient fractionation. As shown in Fig. 2, the sedimentation of lysosomes was markedly enhanced in acidic conditions. The yield of recovery of these organelles in the $1500 \times g$ supernatant decreased from 65% at neutral pH down to 20% at pH 5.4, while the sedimentation of plasma membranes was not significantly modified. About 60% were recovered in the supernatant at pH 5.4 compared to 70% at pH 7.4. Thus, the use of acidic conditions during the $1500 \times g$ centrifugation step enabled a pre-fractionation of the plasma membranes from the lysosomes in the homogenate to be achieved.

The acidic $1500 \times g$ supernatant was then ultracentrifuged over a Percoll gradient at neutral pH. The profiles of distribution of plasma membranes and organelles in the collected fractions were the same as those described above in Fig. 1. Their characterization with radioactivity and enzyme markers are given in Table I. Plasma membranes were collected in fractions 2 and 3. They were enriched 3-fold based on ConA

TABLE I

Specific activities of markers in different fractions of Percoll gradient after centrifugation of a homogenate from CHO cells

Homogenates from lyzed CHO cells were first sedimented at $1500 \times g$, pH 5.4, in order to remove most of the lysosomes. Then, the obtained supernatant was ultracentrifuged on a Percoll gradient. Results are the mean of four experiments. Numbers in parentheses represent the enrichment ratio which was calculated with respect to the cell homogenate. Specific activities are expressed in dpm/mg protein (a), nmol/h per mg protein (b), mmol/min per mg protein (c), μmol/min per mg protein (d). (n.d., not detected).

Markers	Homogenate	Fractions 2 and 3	Fraction 9	Fraction 10
^{3}H-ConA (a) (plasma membranes)	2700 ± 250	8100 ± 300 (3)	100 ± 15 (0.04)	80 ± 10 (0.03)
β-D-Glucosaminidase (b) (lysosomes)	360 ± 20	111 ± 20 (0.3)	70 ± 14 (0.2)	72 ± 8 (0.2)
NADH diaphorase (c) (endoplasmic reticulum)	18 ± 3	9 ± 1 (0.5)	115 ± 20 (6.4)	15 ± 3 (0.8)
Succinate dehydrogenase (d) (mitochondria)	25 ± 5	n.d.	25 ± 4 (1)	425 ± 20 (17)

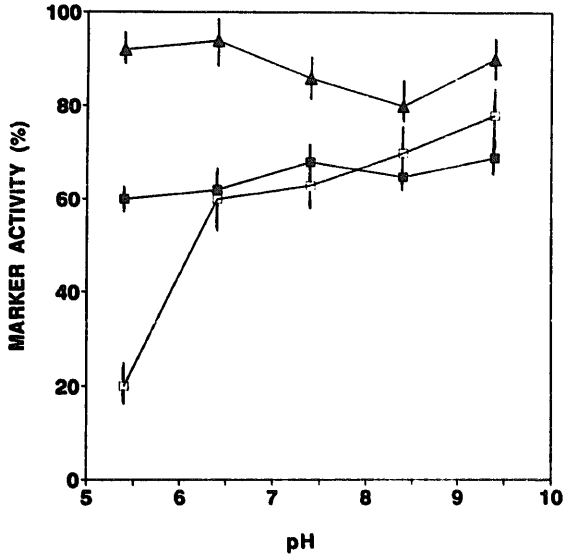

Fig. 2. Effect of the lysis pH on the lysis rate (▲) and on the sedimentation of plasma membranes (^{3}H-ConA radioactivity, ■) and of lysosomes (β-glucuronidase, □) during the 1500× *g* centrifugation. The percentage of lysis was evaluated by measuring the lactate dehydrogenase activity in the cell homogenate. The percentages of recovery of plasma membranes and lysosomes in the 1500× *g* supernatant were calculated by reference to the homogenate. N_2 pressure was fixed at 10 atm.

radioactivity. In agreement with the results shown in Fig. 2, plasma membranes were only contaminated to a slight extent by lysosomes (lysosomes enrichment ratio = 0.3). Presence of endoplasmic reticulum and mitochondria was also negligible. The heaviest fractions 9 and 10 were mainly enriched in endoplasmic reticulum (6.4-fold) and mitochondria (17-fold), respectively. Plasma membranes and lysosomes were practically absent from these fractions.

It can be seen that the conditions described above enabled plasma membranes to be prepared but also endoplasmic reticulum and mitochondria. These various organelles were well characterized and pure enough to be used for enzymatic studies. Unfortunately, difficulties were encountered in the step of elimination of Percoll from the purified plasma membrane (fractions 2 and 3 in Fig. 1) with consequences on the purification of the lipids which were extracted from these fractions. Firstly, it was difficult to deposit the lipids in a homogeneous way on the TLC plates. Secondly, a smear was systematically observed from the bottom to the top of the chromatograms, perturbing the migration of the lipids and making their identification difficult. This was due to the presence of contaminants extracted from residual Percoll. Attempts to purify these plasma membrane lipid extracts by means of various chromatographic techniques (TLC, Silica and Sephadex G-25 columns) were unsuccessful thus making the use of these lipids and membrane fractions for further investigation of the dynamics and topological distribution of lipids in membranes questionable.

For these reasons, another technique of plasma membrane preparation was assayed.

Preparation of plasma membrane from silica-coated cells

As already described in the Introduction, the principle of this method is to enhance specifically the density of the plasma membranes in order to improve their separation from other organelles by low-speed centrifugation.

The first parameter to be determined was the number of silica-polyanion layers to be fixed on the cell surface in order to sufficiently enhance the density of the plasma membranes. In the case of CHO cells, only one silica-polyanion layer was necessary to obtain good separation from other membranes. This was checked by fractionation of the cell lysate over continuous self-generating Percoll gradients in the conditions described above (data not shown). Coated plasma membranes were recovered in the heaviest density fraction (fraction 10). Note that less than 3% of the ^{3}H-ConA fixed on cells were removed during the coating procedure.

Optimization of the nitrogen pressure. Like Chaney and Jacobson [17], we have chosen to lyse the cells by N_2 cavitation. Provided the N_2 pressure is optimized, there is no disruption of subcellular organelles when using this technique [31]. Nevertheless, for coated cells, the pressure and the equilibration time have to be such as to avoid desorption of the silica-polyanion pellicle from the plasma membrane. Moreover, the cell lysis rate must be around 100% in order to increase the yield of membrane preparation and also because intact cells could cosediment with the coated plasma membrane fragments during the differential centrifugations.

Fig. 3 shows the effect of the applied N_2 pressure on the lysis rate of CHO cells. Equilibration time was set at 10 min. As can be seen, the rate of lysis increased regularly while increasing the N_2 pressure from 0 to 30 atm. The highest lysis rate obtained was 90% ± 7%. Surprisingly, for pressure above 30 atm, the lysis rate decreased to 62%. This might be due to too high N_2 pressure which causes disruption of intracellular organelles and the removal, in the suspension medium, of the lytic enzymes they contain with consequences on the integrity of the plasma membrane preparations. For example, if a lysis of lysosomes occurs, the measurement of the cell lysis rate might be artefactual since the proteases released from lysosomes inactivate enzyme. Because of this, PMSF and EGTA, inhibitors of proteases and phospholipases respectively, were added to the suspension medium for better estimation of the cell lysis yield. In agreement with the above hypothesis, addition of these two components in the lysis buffer increased the measured lysis rate from 62% to 85% for

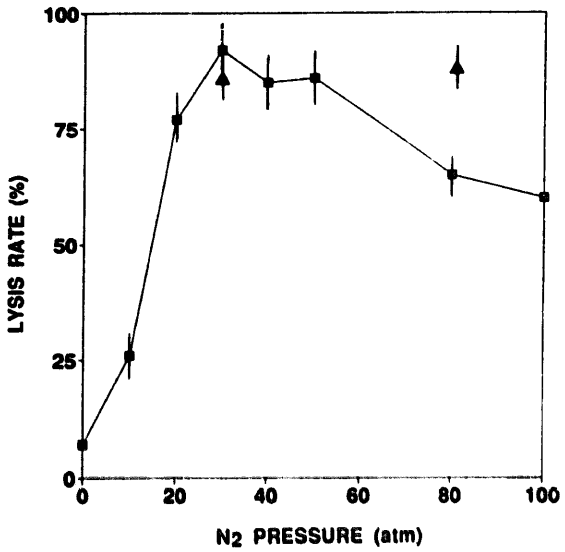

Fig. 3. Effect of the applied N_2 pressure on the lysis rate of CHO cells coated with silica beads. The percentage of lysis was evaluated by measuring the lactate dehydrogenase activity in the cell homogenate (as described in Materials and Methods) without (■) or with (▲) 1 mM PMSF.

a pressure of 80 atm. In any case, maximum cell lysis was obtained for a pressure of 30 atm and this value was chosen as a good compromise for maximal cell lysis and minimal organite lysis.

Optimization of the differential centrifugations. Low-speed centrifugations were then used to fractionate the lysate. A first centrifugation at $200 \times g$ during 3 min removed the small fraction of non-lysed cells. The percentage of ^3H radioactivity recovered in the $200 \times g$ pellet with respect to the total initial radioactivity in the homogenate was identical to the percentage of intact cells found in the lysate before the centrifugation and determined on the ground of lactate dehydrogenase activity. This indicated that no aggregation occurred between cells and plasma membrane sheets during the two steps of cell lysis and $200 \times g$ centrifugation.

The $200 \times g$ supernatant was then fractionated at $400 \times g$ for various periods of time. Results are shown in Fig. 4. The rate of sedimented plasma membranes increased with centrifugation time and then stabilized at 70% for centrifugation times of 20 min and above. Furthermore, for a centrifugation time of 30 min, a non-negligible fraction of nuclei cosedimented with the plasma membrane sheets, as observed in a photonic microscope.

The lack of total recovery of the plasma membranes may be due to the existence of a fraction of uncoated plasma membranes which would not sediment during the low speed centrifugation step. Indeed, ultracentrifugation of the $400 \times g$ supernatant over Percoll gradient as described above revealed the presence of non-coated plasma membrane fragments in fraction 3. These non-coated fractions of plasma membrane might correspond to cell surface regions inaccessible to the silica particles, for example, because of the presence of microvilli. In order to check this hypothesis and with the aim of increasing the coating rate of the plasma membranes, we used the capacity of CHO cells to swell when suspended in an hypotonic buffer. In 50 mM PBS, CHO cells swelled and exhibited a smooth surface as checked by electron microscopy [32] but fixation of silica on these cells did not improve the recovery rate of the plasma membrane. This incomplete coating of the plasma membrane might be due to partial desorption of silica by polyacrylic acid as described by Chaney and Jacobson when dextran sulfate was used [17].

Enzymatic characterization of the plasma membrane fraction

Enzymatic markers were used to evaluate the degree of contamination of the plasma membrane fraction by other organelles. As shown in Table II, the plasma membrane preparations were enriched 7-fold. The concentration of marker enzymes related to the other major organelles were reduced to very low levels.

Biochemical characterization of the isolated plasma membranes

Elimination of the silica-polyanion pellicle was carried out by ultrasonication of the purified plasma membrane fractions in an hypertonic buffer (200 mM PBS). In these conditions, all the pellicle was removed. Indeed, no lipids were detected in Bligh and Dyer extracts of the silica pellets. Furthermore, chemicals

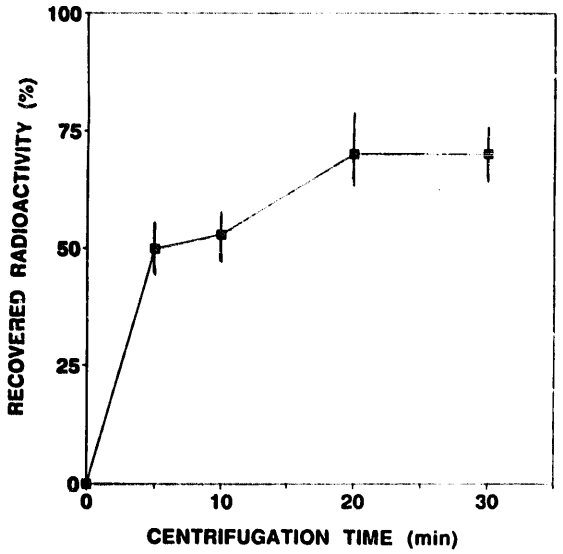

Fig. 4. Influence of the centrifugation time on the sedimentation rate of silica-coated plasma membranes (^3H-ConA radioactivity) during the centrifugation step at $400 \times g$ of cell fractionation. The percentage of radioactivity was calculated by reference to the total radioactivity present in the cell homogenate.

TABLE II

Specific activities of markers in the plasma membrane fractions obtained from silica-coated cells

Results are the means of three experiments. Numbers in parentheses represent the enrichment ratio which was calculated with respect to the cell homogenate. Specific activities are expressed in dpm/mg protein (a), nmol/h per mg protein (b), mmol/min per mg protein (c), μmol/min per mg protein (d).

Markers	Homogenate	Plasma membrane	
^{3}H-ConA (a) (plasma membranes)	4900 ± 100	36000 ± 210	(7.3)
β-D-Glucosaminidase (b) (lysosomes)	580 ± 20	350 ± 20	(0.6)
β-Glucuronidase (b) (lysosomes)	2250 ± 135	1575 ± 107	(0.7)
NADH diaphorase (c) (endoplasmic reticulum)	95 ± 10	33 ± 8	(0.3)
Succinate dehydrogenase (d) (mitochondria)	25 ± 5	2.5 ± 0.5	(0.1)

which are characteristic of the coating pellicle are detected by TLC chromatography of this extract. These compounds were not observed in the lipids extracted from the recovered plasma membrane.

Purified plasma membranes and whole cell membranes were analyzed for their neutral lipid and phospholipid contents. As shown in Table III, the main differences were observed in the cholesterol to phospholipid ratios, which increased from 0.34 in the homogenate up to 0.55 in the plasma membrane.

Table IV shows the phospholipid composition of the total lipid extract from the homogenate and the plasma membrane fractions. It is to be noted that the phospholipid composition of the homogenate is in agreement with those published by Esko and Raetz [33] and by Polokoff et al. [34]. Plasma membranes were enriched in sphingomyelin, in phosphatidylserine and in phosphatidylethanolamine, at the expense of phosphatidylcholine, which decreased from 48% in the homogenate down to 39% in the plasma membrane. Lysophosphatidylcholine and phosphatidylinositol were not detected in the various plasma membrane preparations tested.

TABLE III

Lipid composition of total cellular and CHO plasma membrane extracts

Results are expressed in mol% with respect to the total and are the means of three experiments.

	Homogenate	Plasma membrane
Phospholipids	70 ± 7	62 ± 3
Triglycerides	6 ± 0.5	4 ± 0.5
Cholesterol	24 ± 1	34 ± 2
Chol./Phospholipids	0.34	0.55

TABLE IV

Phospholipids composition of cell homogenates and of plasma membranes isolated from CHO cells

Results are the means of three experiments and expressed in mol% with respect to the total. (n.d., not detected.)

	Homogenate	Plasma membrane
PC	48 ± 2	39 ± 2
PE	24 ± 2	32 ± 2
PI	8 ± 1	n.d.
PS	6 ± 1	11 ± 1
Lyso-PC	4 ± 1	n.d.
SM	10 ± 1	18 ± 1

The possibility of selective binding of these lipids to the silica pellicle can be ruled out since they were not detected in the Bligh and Dyer extracts obtained from the silica pellets and from the silica coated plasma membrane fractions.

Table V reports the fatty acid composition of the phospholipids extracted from the homogenate and the plasma membranes. This composition is in agreement with that described by Rintoul et al. [35]. Only five fatty acids represent more than 95% of the total content. No drastic change in this composition was found between homogenate and plasma membrane lipid extracts. The bi-unsaturated fatty acid 18:2 (linoleic acid) decreased from 11% in the homogenate down to 5% in the plasma membrane to the benefit of all the other species. However, it should be noted that in the plasma membranes, the unsaturated fatty acids represented 53% of the total instead of 58% in the homogenate.

The percentage of saturated fatty acids in phosphatidylcholine and phosphatidylethanolamine was high (70%) compared to that of total plasma membrane phospholipids (42%) (Table V). Insufficient sphingomyelin was recovered to allow direct analysis of its fatty acid content. However, the conditions of alkaline methanolysis used for the analysis of the total or plasma membrane lipid extracts were checked to be strong enough to cleave the amide linkage of sphingomyelin.

TABLE V

Fatty acids composition of phospholipids extracted from cell homogenates and from plasma membranes purified from CHO cells

The data are the mean of two membrane preparations and expressed in percent with respect to the total. (n.d., not detected.)

	Homogenate	Plasma membrane	PC	PE
16:0	26 ± 2	28 ± 3	50 ± 6	41 ± 5
16:1	5 ± 1	5 ± 1	6 ± 1	6 ± 2
18:0	16 ± 1	19 ± 2	17 ± 2	28 ± 4
18:1	42 ± 5	43 ± 2	27 ± 3	24 ± 2
18:2	11 ± 1	5 ± 2	n.d.	n.d.

In these conditions, the fatty acid composition of sphingomyelin can be calculated from that of phosphatidylcholine, phosphatidylethanolamine and the total lipid extract from the plasma membrane. By this means, sphingomyelin was estimated to be acylated mainly by oleic acid (78%), with only 12% saturated fatty acid.

Discussion

To further investigate the composition and the topological distribution of phospholipids in the plasma membrane of CHO cells, obtaining purified plasma membrane fractions, having good yield, high degree of purification and available as rapidly as possible was an obligatory step.

Various methods of fractionation of the membranes of CHO cells have been published [33,36–46] but only a few are relative to the plasma membrane [37,40,41, 43,46] and the data described did not meet the above requirements. Simoni and co-workers have studied the intracellular transport of cholesterol [37,41] or phosphatidylcholine [40] from the endoplasmic reticulum to the plasma membrane of CHO cells. In the first report, plasma membranes were prepared by fixation of cells on DEAE-Sephadex beads. An enrichment ratio of 10 was reported for the plasma membrane but lysosomes were also enriched 2-fold [37]. The yield did not exceed 10%. In further publications, a sucrose gradient was used for membrane fractionation, but no further characterization of the plasma membrane fraction was described [41].

Mackinnon and Mountford [43] have used, without adaptation of the various parameters for CHO cells, a method published by May and co-workers [47] for fractionation of blood lymphocytes and using sucrose gradient. No characterization of the CHO plasma membrane fractions, in terms of yield, enrichment ratio and separation from other organelles, was given. Since these preparations were not clear enough, we have tested other methods of preparation of plasma membranes.

In the two methods which we describe, lysis of CHO cells was carried out using nitrogen cavitation [31]. This technique has the advantage of disrupting plasma membranes with very good yield (about 90%) while preserving intracellular organelles. This point is of particular importance with regards to the lysosomes which are liable to liberate phospholipases and proteases which then might alter the lipid and protein components of the plasma membrane and other cellular organelles. In any case, EGTA and PMSF were added to the lysis buffer in order to prevent the activity of such enzymes, if present, during the various steps of membrane fractionation.

As a first approach, fractionation of plasma membrane and organelles from CHO cells was carried out using ultracentrifugation on a self-generating Percoll gradient. Such a technique was successfully applied for the preparation of the plasma membranes from various cells [11–16]. In the case of CHO cells, the plasma membrane fractions recovered in fraction 3 of the Percoll gradient were well separated from reticulum endoplasmic (fraction 9) and mitochondria (fraction 10) but lysosomes were also found to sediment in the plasma membrane fraction. Attempts to improve the separation by modifying the density of either plasma membranes or lysosomes were unsuccessful.

It has also been described that varying the pH of the Percoll gradient reduced the contamination of the plasma membranes by intracellular organelles, mostly endoplasmic reticulum [12–14]. In our case, decreasing the pH from 7.4 down to 5.4 had no effect on the separation of lysosomes and plasma membranes on the Percoll gradient. In contrast, decreasing the pH of the lysis buffer and subsequently of the first centrifugation step ($1500 \times g$, 10 min) down to 5.4 enabled 80% of the lysosomes to be pelleted. Note that in these conditions, the yield of plasma membrane was also slightly decreased since only 60% were recovered. When using this acidic pH of 5.4 for the cell lysis and prefractionation steps and then a neutral pH for the centrifugation step on the Percoll gradient, plasma membrane fractions enriched 3-fold were collected, practically free of lysosomes. Pure endoplasmic reticulum and mitochondria fractions were also recovered.

In the course of the preparation of this article, it has been reported that separation of plasma membranes and lysosomes from CHO cells could be achieved using Percoll gradient of low density [46]. Nevertheless, no mention was made of the purity and yield of these plasma membrane fraction, nor of the fractionation of mitochondria and endoplasmic reticulum [46].

It is clear that the various membrane fractions so obtained can be conveniently used for many biological tests such as the measurement of enzyme activities. On the other hand, the difficulties encountered in the step of elimination of Percoll from the plasma membrane fractions would prevent them from being used for further investigation of the dynamics and topological distribution of lipids in the plasma membrane of cells. In particular, the Percoll contaminant which was found to systematically affect the lipids extracted from these plasma membrane fractions would prevent the identification and counting of the various lipid dimers which are formed when using the photodimerization method for studying the lateral distribution of lipids in membranes [10]. Furthermore, it was also observed that this contamination prevented the phospholipids from forming well organized multilayers on microscope slides for further FRAP experiments [30].

To circumvent these drawbacks, we tested an interesting procedure which has already been successfully

applied to the preparation of the plasma membranes from *Dictyostelium discoideum* [17], plant cells [48] or apical and basolateral domains of plasma membranes from HELA [49,50], MDCK [51] and vascular endothelial [52] cells. It is based on the increase of the plasma membrane density by fixation on the cell surface of cationic silica particles stabilized by polyacrylic acid. After disruption of the cells, plasma membranes are easily recovered by differential centrifugations. One major advantage of this technique is its simplicity and rapidity. By differential centrifugations, plasma membrane fractions enriched 7-fold and practically free of intracellular organelles were obtained with a final recovery yield of 70%, within less than 1 h. It is difficult to evaluate the purity of these membrane fractions precisely simply using the enrichment ratios shown in Table II. Nevertheless, it has already been reported, on the grounds of electron microscopy, that CHO cells are poorly endocytotic and relatively poor in intracellular organelles, in particular in mitochondria [9]. If one assumes that the plasma membrane in CHO cells constitute 15–20% of the total cell membranes, and taking a mean relative plasma membrane to organelles enrichment ratio of 30 (see Table II), the purity of the plasma membrane fractions so obtained can be roughly estimated at around 85–90%.

In addition to time saving and better enrichment ratio, a great advantage of the second method was that the silica-polyanion pellicle was easily and totally eliminated, by ultrasonication and centrifugation, from the recovered plasma membrane fractions, thus enabling lipids to be extracted free of any contamination.

With respect to the lipid composition, a relative decrease in the phospholipid content and a relative increase in the cholesterol content of the plasma membrane, as compared to the cell homogenate, were observed. This yielded a cholesterol to phospholipid ratio of 0.55 for the plasma membrane, which is quite usual for this cell organite [53] and which provides further support to the above conclusion of a high purity for these plasma membrane preparations. With respect to the phospholipids, the plasma membrane of CHO cells comprised mainly phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylserine. Phosphatidylinositol and lysophosphatidylcholine, which were identified in the total cell lipid extract, were practically absent from the plasma membrane. Such a phospholipid composition is very similar to that reported for the plasma membrane of other mammalian cells [53].

Finally, cell homogenate and plasma membrane lipid extracts exhibited rather similar fatty acid composition, with the exception of linoleic acid which was less abundant in the plasma membrane than in the total cell lipid extract. Phosphatidylcholine and phosphatidylethanolamine displayed a rather simple fatty acid composition with only palmitic, palmitoleic, stearic and oleic acids. If significant differences were found in the relative distribution of these four fatty acids between the two lipids, the saturated to unsaturated fatty acid ratios were very similar, to a value of around 2.1. As mentioned in Results, and from the comparison of the fatty acid composition of phosphatidylcholine, phosphatidylethanolamine and of the total lipid extract from the plasma membrane, it was estimated that sphingomyelin was mainly acylated by oleic acid (78%) with only 12% saturated fatty acids. Such large differences in the saturated to unsaturated fatty acid ratios between the two major lipids phosphatidylcholine and phosphatidylethanolamine on the one hand, and sphingomyelin on the other hand, might possibly be related to our previous observation of the existence of lipid microdomains in the plasma membrane of CHO cells [9].

It should be stressed that the yield of plasma membrane with the silica coating method was always 70 ± 10%. Chaney and Jacobson have reported similar results (70–90%) [17]. We have checked whether this non-coated plasma membrane fraction of about 30% might originate from plasma membrane regions, between microvilli, which might be inaccessible to silica. Apparently, this was not the case since the coating of smooth CHO cells, after swelling in water, gave similar plasma membrane yield. We are still unaware of the origin of these non-coated plasma membrane fractions and the possibility that they correspond to peculiar cell surface regions is not to be ruled out a priori. Care should be taken when interpreting topological data obtained with plasma membrane fractions purified by this method.

Finally, it is worth emphasizing that the stabilization of plasma membrane by silica-polyanion generates membrane fragments in the form of open sheets [17] which are coated and therefore protected only on the external face, thus allowing an asymmetric action of any chemical or biochemical reagent. Chaney and coll. have used this property to study the transverse distributions of proteins in plasma membrane of various cellular systems [17,48,50,51]. Similarly, the use of phospholipases or of non-penetrating chemical reagents on these protected plasma membrane fragments would enable the transverse distribution of phospholipids to be determined in the plasma membrane of CHO cells. This study is currently under investigation in our laboratory.

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