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LM FIBROBLAST PLASMA MEMBRANE SUBFRACTIONATION BY AFFINITY CHROMATOGRAPHY ON CON A-SEPHAROSE

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Affinity chromatography was used to determine the heterogeneity and orientation of plasma membrane vesicles isolated from LM fibroblasts subjected to Dounce homogenization. Two plasma membrane subfractions were obtained by Con A-Sepharose affinity chromatography of LM fibroblast plasma membranes prepared by Dounce homogenization. The desmosterol-phospholipid molar ratio, the phospholipid composition, and the phospholipid fatty acid composition were almost identical between the two fractions. However, the lipid to protein ratio was almost 2-fold greater in the nonadherent fraction A. The binding of fluorescein-concanavalin A was the same in both fractions indicating a right-side-out orientation of the vesicles. Similarly the asymmetric distribution of phosphatidylethanolamine in both membrane fractions was the same. In contrast, sialic acid content, 5′-nucleotidase activity, and ($Na^+ + K^+$)-ATPase activity were 47%, 3.7-fold, and 2.5-fold greater, respectively, in the nonadherent, lipid-rich fraction A. Structural properties of the two membrane fractions determined by fluorescence polarization and Arrhenius plots of *trans*-parinaric acid fluorescence were similar. These results indicate that concanavalin-A affinity chromatography separates two membrane fractions differing in sialic acid content, lipid content, and enzyme profile but having the same right-side-out orientation.

Introduction

Plasma membrane vesicles isolated from mammalian cells have become important to approaches in mammalian transport [1], the study of lymphocyte blastogenesis [2–5], the formation of metastatic tumor cells [6], the determination of asymmetric distribution of lipid in membranes [7,8], the demonstration of the existence of fluidity gradients across cell plasma membranes [7–10], and the study of biophysical properties of cell membranes [7–13]. Much of this work assumed that the plasma membrane vesicles isolated from mammalian cells were uniform in composition and properties. However, recent results from many laboratories using affinity chromatography (e.g. insulin-agarose [14], H-2 antibody-agarose [15], Con A-Sepharose [2,3] and nicotinic receptor ligand-agarose [16]), electrophoresis of lymphocyte plasma membrane vesicles [5], ionic lysis of red blood cells [17], or gradient centrifugation of intestinal microvillus membrane vesicles [18] indicated that plasma membrane vesicles could be subfractionated. These findings led to a host of new questions regarding the origin of these different membrane vesicles: do they represent functional and structural mosaicism in the cell surface; are they membrane fractions from heterogeneous cell types; do they represent inside-

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out or right-side-out oriented membrane vesicles; do they represent plasma mebrane vesicles that are dependent on the cell cycle; etc. The answers to these questions have been slow in coming and have been complicated by the fact that heterogeneous cell types or tissues have been used in much of this work. Lipid analyses and biophysical characterizations were also not reported. Herein are presented the results of lipid composition, enzyme profile, and structural analyses of the two subfractions obtained after affinity chromatography on Con A-Sepharose from LM fibroblast plasma membrane vesicles. The LM fibroblast represents a single cell type cultured in a chemically defined medium.

Materials and Methods

Cell culture and plasma membrane isolation

LM cells, a choline-requiring strain of mouse fibroblasts, were cultured in suspension in a serum-free, chemically defined medium [19]. Cells growing in logarithmic phase were centrifuged at $225 \times g$ for 10 min and resuspended in medium containing 2 μCi $^{32}P_i$/ml (New England Nuclear, Boston, MA). The cells were cultured at $1 \cdot 10^6$ cells/ml in medium of the same specific activity for 6 days. This procedure labeled all the phospholipids to uniform specific activity. Lipid compositions determined by ^{32}P or by phosphate analysis were the same. Plasma membranes were isolated from the LM cells as described by Schroeder et al. [19].

Trinitrobenzenesulfonic acid labelling reaction

The asymmetric distribution of phosphatidylethanolamine in LM cell membranes can be determined by using the chemical labelling reagent, trinitrobenzenesulfonic acid, which binds covalently to primary amines of phospholipids and proteins [7,8,20–22]. The cells or isolated membrane vesicles were treated with 4 mM trinitrobenzenesulfonic acid under nonpenetrating conditions, pH 8.5, at 4°C for 80 min, as described by Fontaine and Schroeder [20]. Penetration of the reagent into the cells was monitored by determination of intracellular labelling of microsomal and mitochondrial lipids.

Plasma membrane subfractionation on Con A-Sepharose

Plasma membrane vesicles were subfractionated by a modification of the procedure of Brunner et al. [3]. Buffer A (pH 7.2, 24°C) consisted of 0.14 M KCl, 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid (Hepes), 1 mM $MgCl_2$, and 1 mM $MnCl_2$. A 2×30 cm glass column with a sintered glass filter was rinsed with 50 ml of buffer A. 16 ml of Con A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) were washed three times with buffer A, by centrifugation at $200 \times g$ for 5 min. The plasma membrane fraction from $500 \cdot 10^6$ cells was warmed to 24°C by swirling the container in 24°C water and then added to the 16 ml of Con A-Sepharose 4B and incubated for 15 min. The solution was then mixed by bubbling nitrogen lightly through the suspension for 2 min and then allowed to stand undisturbed for 13 min. The suspension was then gently poured into the column and allowed to stand undisturbed for another 5 min. The column was eluted with 16 ml buffer A at a rate of 2 ml/min and 2-ml fractions were collected. These were called A_1 fractions. Then a second 16-ml aliquot of buffer A was added and fractions A_2 were collected. Fractions A_3 were collected similarly after adding another 16 ml of buffer A. A 0.1-ml aliquot was taken from each 2-ml fraction and the ^{32}P radioactivity determined by liquid scintillation counting to obtain an elution profile. With the column stopcock closed, 16 ml of buffer B (pH 7.2, 23°C) (which consisted of buffer A containing 0.5 M α-methylmannoside) was added to obtain the fraction B vesicles. Nitrogen was bubbled vigorously through the column bed via a Pasteur pipette for 13 min. Then while nitrogen was bubbled slowly through the column bed, 2-ml fractions were collected at a rate of 2 ml/min. These were called B_1 fractions. The two 16-ml portions of buffer B were added to collect B_2 and B_3 fractions and 0.1-ml aliquots were taken for scintillation counting. 16 ml of buffer C consisting of 1.0 M NaCl (pH 7.0, 23°C) was used to elute any remaining vesicles in one 16-ml fraction (C_1). All of the elution procedures were performed at room temperature (24°C) under a N_2 atmosphere. After elution from the Con A-Sepharose column, the peak fractions were combined and sedimented for 1.5 h at 39000 rpm on a

Type 40 rotor with an L5-65 Ultracentrifuge (Beckman Instr., Fullerton, CA). The resulting pellet was resuspended in 10 ml physiological saline, pH 7.0, containing 1 mM triethanolamine and again sedimented. This procedure removed the eluting sugars.

Permeability of plasma membrane vesicles during reaction with trinitrobenzenesulfonate

Permeability of vesicles was tested in two ways: First, if the plasma membrane vesicle subfractions obtained by Con A-Sepharose chromatography were leaky, then trinitrobenzenesulfonic acid (TNBS) would penetrate into the vesicles and label phosphatidylethanolamine and other primary amines [20–22]. Therefore, plasma membranes and subfractions A and B were exposed to 4 mM TNBS at 4°C as described earlier [20]. In leaky vesicles up to 90 + % of the membrane phosphatidylethanolamine was labeled. In contrast, in the present investigation only 20 ± 5% of the phosphatidylethanolamine was trinitrophenylated in unfractionated membrane vesicles, 24 ± 6% in subfraction A, and 21 ± 4% in subfraction B. Second, the integrity of the vesicles was determined by efflux of [^{3}H]thymidine as follows: 200 ml of LM cells previously grown for 6 days on $^{32}P_i$ were washed as described above. The cells were resuspended with 1 μCi [^{14}C]thymidine (New England Nuclear Inc., Boston, MA) and the plasma membranes were prepared and resuspended in 1 ml of phosphate-buffered saline [19]. The vesicles were washed two times by dilution (1:10) with phosphate-buffered saline and centrifuged at 39000 rpm on a type 40 rotor (Beckman Instr., Fullerton, CA). The ratio of ^{14}C to ^{32}P in the washed, isolated plasma membrane vesicles was 0.1 ($1.76 \cdot 10^6$ cpm total counts). The vesicles were split into two fractions and incubated with one fraction at 24°C and the other at 4°C for 80 min. The reaction was terminated by Millipore filtration using 1 μm glass filters (Gelman Glass Filter, Type A-E) under reduced pressure. The leakiness of the plasma membrane vesicles was expressed as the ratio (^{14}C in the filtrate/^{14}C on filter) $\times 100$. Vesicles passing through the filter were estimated by ^{32}P-labeled phospholipid in the filtrate. More than 99.9% of the ^{32}P was found in the filter indicating that the intact vesicles stayed in the filter. Less than 5.6% of trapped [^{14}C]thymidine leaked out of fraction A, B, or unfractionated membrane vesicles in 80 min at 24°C. Lastly, in order to demonstrate that [^{14}C]thymidine was not binding to the vesicles but rather actually trapped, another preparation of plasma membranes containing no [^{14}C]thymidine was mixed with $0.17 \cdot 10^6$ cpm [^{14}C]thymidine and subsequently passed over the filter. Over 99.9% of the thymidine appeared in the filtrate indicating very little binding to either membranes or the filter.

[^{3}H]Ouabain binding

The binding of [^{3}H]ouabain was determined by the method of Hitzeman [23], except that preincubation of the fractions with ATP was increased from 2 to 4 min, and the incubation with [^{3}H]ouabain was increased from 5 to 10 min. About 100000 counts were incubated with 100 μg membrane protein. This gave a final [^{3}H]ouabain concentration of $2 \cdot 10^{-8}$ M. Buffer D contained 40 mM NaCl, 20 mM Tris (pH 7.4), 0.25 mM EDTA, 3 mM $MgCl_2$. This buffer D was used to determine the amount of non-specific binding of the ouabain, since the phosphorylated intermediate of the ATPase enzyme is required for binding. Buffer E was composed of buffer D plus 3 mM ATP. The incubation was conducted directly in Minivial liquid scintillation vials (Research Products International, Elk Grove, IL). It was important that the Minivial not be vortexed since this exposed more surface area of the Minivial to the [^{3}H]ouabain and increased the background counts from about 200 to 1000–1300 cpm. Instead, 100 μl of the buffer containing 100000 cpm [^{3}H]ouabain (New England Nuclear, 19 μCi/mmol) was delivered into the center of the vial, and then the test tube rack of vials was gently shaken for 20 s.

After incubating the membranes with [^{3}H]ouabain-ATP, the Minivials were centrifuged in an SS-24 Sorvall rotor for 15 min at 17000 rpm ($35000 \times g$). The Minivials collapse at greater g forces. The supernatant was aspirated, the surface of the pellet was washed with 1 ml of cold buffer E minus [^{3}H]ouabain, and aspirated again. 5 ml of Budget Solve (Research Products International, Elk Grove, IL) was added, and counts/min were determined.

Lipid analysis

Lipids were extracted and separated by silicic acid chromatography as described earlier [19]. The phospholipids were separated by two-dimensional chromatography, identified, and quantitated as described by Fontaine and Schroeder [20]. Phospholipids were eluted from thin-layer plate scrapings as follows: Each gram of silica gel was eluted twice with $CHCl_3/CH_3OH/1.5\%$ NH_4OH (6:5:1, v/v) and twice with CH_3OH. Recoveries were approx. 95%. Fatty acid methyl esters of phospholipids were prepared using BF_3/CH_3OH and analyzed using a Sigma 2 Gas Chromatograph (Perkin-Elmer Norwalk, CT) with a HP 3390 Reporting Integrator (Hewlett-Packard Inc., Avondale, PA) on a 10% SP-2340 on 100/120 Chromosorb WAW column by temperature programming (Supelco, Inc., Bellefonte, PA). The fatty acid methyl esters were identified by comparison of R_F values with authentic standards: methyl palmitate, methyl palmitoleate, methyl linoleate, and methyl linolelaidate (Supelcor, Inc., Bellefonte, PA and Nu-Chek Prep., Elysian, MN). The neutral lipid fraction from silicic acid chromatography was analyzed for sterol content by gas chromatography on a 3% SE-30, 80/100 mesh, Chromosorb WHP column at 235°C (Pierce Chemical Co., Rockford, IL). LM fibroblasts synthesize desmosterol instead of cholesterol. Desmosterol was identified by comparison of retention times with authentic standards of desmosterol, cholesterol, and coprostanol obtained from Steraloids Inc., Rawling, NY.

Flurorescence measurements

trans-Parinaric acid was incorporated into LM cell membranes and subfractions as described by Schroeder et al [12,13] at a probe/lipid molar ratio of 1:100 or less. Absorption, absorption-corrected fluorescence and relative quantum efficiency were determined as previously described [12,13] using the computer centered spectrofluorimeter developed by Holland et al. [24,25]. Light scattering was corrected by use of appropriate cutoff filters and narrow bandpasses. The fluorescence was computer corrected for instrumental and inner-filter effect variables [24,25]. Fluorescence polarization was measured with a T format SLM 4800 Subnanosecond Spectrofluorometer (SLM Instr. Inc., Champaign-Urbana, IL) containing Glan-Thompson Polarizers in the excitation and emission light beams. Light scattering was reduced by use of narrow bandpasses and cutoff filters. The fluorescence polarization was measured as the limiting polarization at zero absorbance. Excitation and emission were determined at 313 and 415 nm, respectively.

Analytical procedures

Lipid phosphorus analysis was determined as described by Ames [26]. Ouabain-sensitive ($Na^+ + K^+$)-ATPase and 5′-nucleotidase activity were determined as described by Schroeder et al. [19]. Sialic acid was assayed by the method of Warren [27]. Protein was determined by the method of Lowry et al. [28]. The plasma membrane subfractions eluted from the column contained displacing sugars which might interfere with the Lowry protein assay. These sugars were removed by wash procedures as described above and did not interfere with protein determinations in the present work. FITC-concanavalin A binding was determined as described by Monsigny et al. [29].

Results

Subfracionation and characterization of LM plasma membrane vesicles

Plasma membrane vesicles obtained from tissues such as liver or from blood-borne cells such as lymphocytes or macrophages represent membrane fractions derived from different cell types. Therefore, the source for the plasma membrane vesicles used herein was a single cell type, LM fibroblasts, cultured in a chemically-defined, serum-free medium. The plasma membrane vesicles isolated by conventional methods, such as Dounce homogenization or nitrogen cavitation with subsequent gradient centrifugation, represent a family of membrane vesicles derived from the cell surface membrane. In an attempt to determine if subfractions of LM cell plasma membranes have a homogeneous or heterogeneous carbohydrate composition, lipid composition, enzymatic activity, or asymmetric distribution of physical characteristics, the plasma membranes isolated as described in Methods were subfractioned by Con A-Sepharose chromatography (Fig. 1). Two fractions were

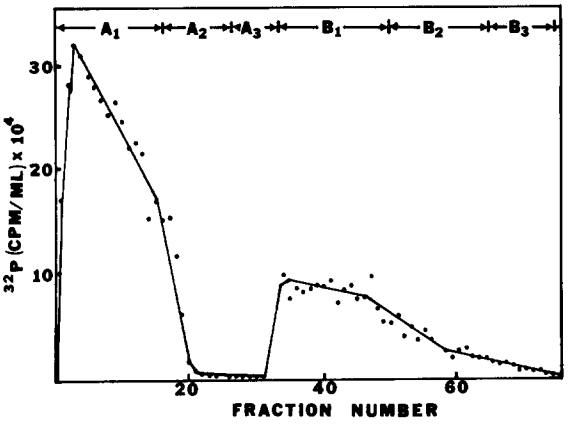


Fig. 1. Con A-Sepharose affinity chromatography of plasma membranes isolated from whole LM cells. Con A-Sepharose (20 ml/5 mg plasma membrane protein) was washed 4 times with 4 vol. buffer A (140 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, 1 mM $MnCl_2$). The plasma membrane sample was then incubated for 15 min at 24°C with Con A-Sepharose. The Con A-Sepharose plus sample was then placed in a 2 cm×30 cm column. Three 16 ml portions of buffer A were then added sequentially to obtain fraction A. Three 16 ml portions of buffer A containing 0.5 M α-methylmannoside were then added to obtain fraction B.

eluted. Fraction A which did not bind to the column contained 56.3 ± 2.2% ($n = 3$) of the eluted phospholipid; while the adherent fraction B contained 33.7 ± 2.1% ($n = 3$) of the eluted phospholipid (Table I). Similar values were determined by lipid phosphate analysis. Recoveries from the column were 90 ± 3%. Several surface membrane markers were determined to characterize the membrane subfractions (Table I) (1) Sialic acid residues are the primary negative charge determinant of cell surfaces and are, therefore, enriched in plasma membranes. The total sialic acid content of fraction B was 32 ± 1% lower than that of fraction A. The value for unfractionated plasma membrane vesicles was intermediate. (2) The sialic acid content of LM fibroblast membranes was about 3–4-times higher than that reported for mouse L cells [30]. (3) Unfractionated plasma membrane vesicles from LM fibroblasts were enriched 7.2-fold and 8.3-fold, respectively, in 5′-nucleotidase and ($Na^+ + K^+$)-ATPase activity as compared to the crude homogenate. However, the activities of these enzymes were unequally distributed between the subfractions. The specific activity of 5′-nucleotidase in fraction B was 73% lower than in fraction A while the activity was intermediate in the unfractionated plasma membrane. Similarly, the specific activity of ($Na^+ + K^+$)-ATPase activity in fraction B was 60% lower than in fraction A. Some loss of ($Na^+ + K^+$)-ATPase occurred on the column since the normalized sum of the activities in the two fractions did not add up to that in the unfractionated plasma membrane. Under the assay conditions used herein, the activities of 5′-nucleotidase, of ($Na^+ + K^+$)-ATPase, and the content of sialic acid reflected the total activity or content (both the outer surface and the inner surface of the membrane vesicles were exposed to the reagents). These results indicate that the two plasma membrane subfractions were heterogeneous with respect to surface membrane markers.

It is possible that during the homogenization procedure right-side-out and inside-out plasma membrane vesicles may be produced. The activity

TABLE I

SURFACE MARKER ACTIVITIES OF LM CELLS PLASMA MEMBRANE SUBFRACTIONS

Sialic acid was determined by the method of Warren, L [27]. 5′-Nucleotidase and ($Na^+ + K^+$)-ATPase were determined as described by Schroeder et al. [19]. Values represent the mean ± S.E. ($n = 3$).

Membrane fractions	% of total phospholipid	Sialic acid (nmol/mg protein)	5′-Nucleotidase (nmol/min/mg protein)	($Na^+ + K^+$)-ATPase (nmol/min/mg protein)
Unfractionated plasma membrane	100	62.4	22.5 ± 1.5	63.8 ± 2.1
Fraction A	56.3 ± 2.2 [a]	79.7 ± 0.4 [a]	26.0 ± 1.8 [a]	50.8 ± 0.3 [a]
Fraction B	33.7 ± 2.1 [a]	54.4 ± 0.5 [a]	7.0 ± 1.0 [a]	20.7 ± 1.4 [a]

[a] $P < 0.01$ between fractions A and B.

TABLE II

ORIENTATION OF LM PLASMA MEMBRANE SUBFRACTIONS

Concanavalin A binding was determined with FITC-concanavalin A by the method of Monsigny et al. [29] and are expressed as specifically bound corrected fluorescence (F_{co}) per mg membrane protein. [^{3}H]Ouabain binding was measured by the method of Hitzemann [23] as described in Materials and Methods.

Membrane fractions	FITC-Con A binding (F_{co}/mg protein)	[^{3}H]Ouabain binding (pmol/mg protein)
Unfractionated plasma membrane	291 ± 9	274 ± 22
Fraction A	157 ± 2	195 ± 15 [a]
Fraction B	153 ± 12	124 ± 9 [a]

[a] $P < 0.01$ ($n = 3$).

of surface markers such as [^{3}H]ouabain binding and fluorescein-concanavalin A binding should be much higher in right-side-out than in inside-out vesicles since receptors for concanavalin A and for ouabain are located on the extracellular face of the plasma membrane. In order to measure these activities properly the vesicles must be sealed. The permeability of these membrane fractions A and B was determined as described in Materials and Methods. Less than 5.6% of trapped [^{14}C]thymidine leaked out of fraction A, B or unfractionated membrane vesicles in 80 min at 24°C. In addition, only 20% of the plasma membrane phosphatidylethanolamine was available for reaction with trinitrobenzenesulfonic acid in the membrane subfractions (see Materials and Methods). Thus, both the unfractionated plasma membrane vesicles and fractions A and B were sealed. This result is consistent with that of others [1] who isolated sealed L cell plasma membrane vesicles by a similar procedure.

Concanavalin A binds to specific surface carbohydrate residues [31]. Fraction A and B bound equal amounts of fluorescein-concanavalin A (Table II). However, the binding was reduced as compared to unfractionated plasma membranes. It should be noted that fraction A vesicles contained almost 2 times as much lipid/mg protein as fraction B vesicles (Table III). Thus, fraction B vesicles contained two times as many concanavalin A receptors on the basis of total vesicle mass (protein + lipid). This, as well as size and charge differences in vesicle population, may account for the separation achieved on Con A-Sepharose. Lysed vesicles (in distilled water) did not bind more fluorescein-concanavalin A. Thus, the subfractions A and B both appeared to have a right-side-out orientation. Consistent with this finding were the results obtained with [^{3}H]ouabain binding. As expected from the ($Na^+ + K^+$)-ATPase activity (Table I) fraction A bound more [^{3}H]ouabain than did fraction B. Lysed membrane vesicles did not bind additional [^{3}H]ouabain. Thus, LM plasma membrane subfractions A and B appeared to have a right-side-out orientation.

Lipid composition of LM plasma membrane subfractions

The desmosterol/protein and phospholipid/protein ratios were 44 ± 7% and 47 ± 5% lower, respectively, in fraction B as compared to fraction A vesicles (Table III). However, the desmosterol/

TABLE III

LIPID COMPOSITION OF PLASMA MEMBRANE FRACTION FROM LM SUSPENSION CELLS

Plasma membranes fraction A and B were prepared as described in Methods. Values refer to mean ± S.E. ($n = 3$).

Plasma membrane fraction	Desmosterol/protein (nmol/mg)	Phospholipid/protein (nmol/mg)	Desmosterol/phospholipid (nmol/nmol)
Fraction A	371 ± 25 [a]	588 ± 12 [a]	0.63 ± 0.09
Fraction B	208 ± 26 [a]	309 ± 32 [a]	0.70 ± 0.15

[a] $P < 0.05$ between fractions A and B.

TABLE IV

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM LM SUSPENSION CELL PLASMA MEMBRANE FRACTIONS

Plasma membranes were isolated and fractionated as described in Methods. The phospholipids were purified by silicic acid chromatography and fatty acid methyl esters were prepared as described in Methods. L/S refers to the ratio of long (C>18) to short (C<18) carbon fatty acids. Similarly, U/S refers to the ratio of unsaturated to saturated fatty acids. Values represent the mean ±S.E. ($n=3$).

Fatty acid	% composition	
	Fraction A	Fraction B
14:0	0.4±0.4	0.7±0.4
15:0	0.4±0.1	0.3±0.1
16:0	15.5±0.6	15.6±2.8
16:1	6.0±0.4	5.8±0.2
18:0	11.4±1.5	12.8±2.1
18:1	45.3±0.6 [a]	53.0±1.9 [a]
18:2	1.4±1.1	0.7±0.2
>18	18.6±3.9	10.5±0.6
L/S	3.4	3.5
U/S	1.9	2.0

[a] $P<0.05$ between fractions A and B.

phospholipid ratio was not altered. The latter result is similar to that reported with lymphocytes by Brunner et al. [3]. These data indicate that fraction B contained more protein (less lipid) than fraction A. The fatty acid composition of the phospholipids from fraction A and B was similar except for oleic acid (18:1) which was enriched in fraction B (Table IV). The phospholipid composition of fraction A and B did not differ significantly (Table V).

The phospholipids of LM surface membranes are asymmetrically distributed across the membrane bilayer [7,8,20–22,32]. It is possible that the two membrane subfractions may represent different domains with dissimilar distribution of lipids. The elution profile of plasma vesicles from LM cells treated with trinitrobenzenesulfonic acid was very similar to that of untreated cells (Fig. 2). Table VI indicates that, when the whole LM cells were treated with trinitrobenzenesulfonic acid at 4°C and the membrane fractions were subsequently isolated, the asymmetric distribution of phosphatidylethanolamine across the membrane bilayer was similar for both membrane fractions. Little penetration of the labeling reagent into the cells occurred since only small amounts of the microsomal and mitochondrial phosphatidylethanolamine were trinitrophenylated. Likewise, when purified subfractions A and B were exposed to trinitrobenzenesulfonic acid at 4°C, only 24 ± 6% and 21 ± 4% of the phosphatidylethanolamine was trinitrophenylated. These results are consistent with the possibility that the asymmetric distribution of the two membrane subfractions was similar. Also, since phosphatidylethanolamine is primarily located on the inner surface of the LM cell plasma membrane [20], the plasma membrane vesicle subfractions have a right-side-out orienta-

TABLE V

PHOSPHOLIPID COMPOSITION OF PLASMA MEMBRANE FRACTIONS FROM LM CELLS GROWN IN SUSPENSION

Plasma membrane vesicles were fractionated by Con A-Sepharose chromatography as described in Methods. Values represent the mean ±S.E. ($n=3$).

Phospholipid species	Plasma membrane	Fraction A	Fraction B
Phosphatidylcholine	37.3±2.5	35.0±3.0	34.9±2.9
Phosphatidylethanolamine	28.1±1.9	28.6±2.8	27.6±1.5
Phosphatidylinositol	6.5±0.9	7.4±1.4	6.7±1.4
Phosphatidylserine	3.1±1.2	3.5±0.5	3.0±1.2
Sphingomyelin	4.5±0.6	5.9±1.5	5.2±0.9
Phosphatidylglycerol	6.5±2.0	7.2±2.0	6.4±1.5
Lysophosphatidylcholine	8.9±1.7	8.8±1.5	8.7±1.4
Cardiolipin	–	–	–
Other	3.9±1.6	3.6±0.8	7.6±2.

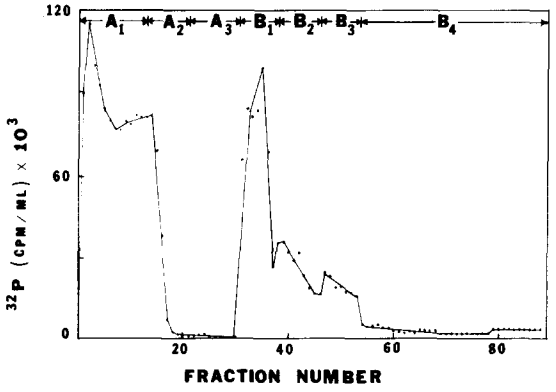


Fig. 2. Con A-Sepharose affinity chromatography of plasma membranes isolated from whole LM cells treated with trinitrobenzenesulfonic acid (TNBS). Whole cells were treated for 80 min with 4 mM TNBS at 4°C. The plasma membrane vesicles were then isolated and passed over a Con A-Sepharose column. Con A-Sepharose (Miles, Elkhart, IN), 20 ml/5 mg protein was washed 4 times with 4 vol. of buffer A (140 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, 1 mM $MnCl_2$). The plasma membrane sample was then incubated for 15 min at 24°C with Con A-Sepharose with occasional light mixing by swirling the flask gently. The Con A-Sepharose plus sample was then placed in a 1 cm×20 cm column. Three 16 ml portions of buffer A were then added sequentially to obtain fraction A. Three 16 ml portions of buffer A containing 0.5 M α-methylmannoside were then added to obtain fraction B.

tion. Isolation of plasma membrane vesicles by the present technique appears to expose additional phosphatidylethanolamine for trinitrophenylation by trinitrobenzenesulfonic acid. In the intact cell

TABLE VI

PHOSPHATIDYLETHANOLAMINE DISTRIBUTION IN PLASMA MEMBRANE FRACTIONS FROM LM FIBROBLASTS

Whole cells were treated for 80 min with 4 mM TNBS at 4°C as described in Methods. The plasma membrane vesicles were isolated and passed over Con A-Sepharose. The columns were eluted as described in Methods. The % phosphatidylethanolamine labeled was also determined as stated in Methods.

Membrane fraction	% Phosphatidylethanolamine labelled
Plasma membrane	2.2
Fraction A	1.8
Fraction B	2.4
Microsomes	3.8
Mitochondria	3.7

apparently 10–15% of the phosphatidylethanolamine in the surface membrane is not accessible to the reagent [20].

Fluorescence probe analyses of LM plasma membrane subfractions

The spectral characteristics of the fluorescence probe molecule, *trans*-parinaric acid, in fraction A and B indicated no major alterations in peak ratios or wavelength shifts. However, fraction A exhibited both a higher corrected fluorescence and a higher relative fluorescence efficiency than either the unfractionated plasma membranes or fraction

TABLE VII

FLUORESCENCE PARAMETERS OF β-PARINARIC ACID IN PLASMA MEMBRANE FRACTIONS OF LM CELL FIBROBLASTS

Plasma membranes were isolated as described earlier [19]. Fraction A and B were obtained after separation of plasma mebrane vesicles on Con A-Sepharose as described in Methods. Membranes (100μg) were incubated in 2 ml phosphate buffered saline and 1μg β-parinarate for 5 min at 30°C as described in Methods. The samples were read at 24°C excitation at 313 nm and emission at 415 nm. values represent the mean ±S.E. ($n=3$). TNP, trinitrophenyl.

Sample	Absorbance	Corrected flurorescence	Relative fluorescence efficiency	Polarization	Limiting polarization with TNP-glycine
Plasma membranes	0.317±0.030	38.0±4.0	52.1±3.5	0.318±0.005	0.350±0.007
Fraction A	0.509±0.060 [a]	49.6±3.0 [a]	68.0±4.0 [a]	0.347±0.006	0.347±0.012
Fraction B	0.392±0.050 [a]	30.5±6.0 [a]	41.8±8.0 [a]	0.355±0.013	0.344±0.009

[a] $P<0.05$ between fractions A and B.

B (Table VII). The average of the corrected fluorescence and relative fluorescence efficiency of *trans*-parinaric acid in fraction A + B (after normalization for the relative amounts of each membrane fraction) was the same as in the unfractionated plasma membranes. Fluorescence polarization of β-parinaric acid was higher in both fractions A and B than in the unfractionated plasma membranes. Therefore, in contrast to the corrected fluorescence and relative fluorescence efficiency, the normalized average polarization of fractions A + B was significantly higher than that in the unfractionated plasma membranes. This may indicate that the membrane structure of the plasma membrane vesicles was altered during their passage through the ConA-Sepharose columns. The polarization of fluorescence emission in each monolayer of the plasma membrane bilayer can be investigated by determining the limiting polarization with trinitrophenyl-glycine (TNP-glycine) in the surrounding fluid [7,8]. The limiting polarization of the unfractionated plasma membranes, fraction A and fraction B were both approx. 0.35 (Table VII).

Other investigators have demonstrated that different membrane areas of LM cell surface membranes may have different lipid phase transition temperatures [9,10]. However, Arrhenius plots of

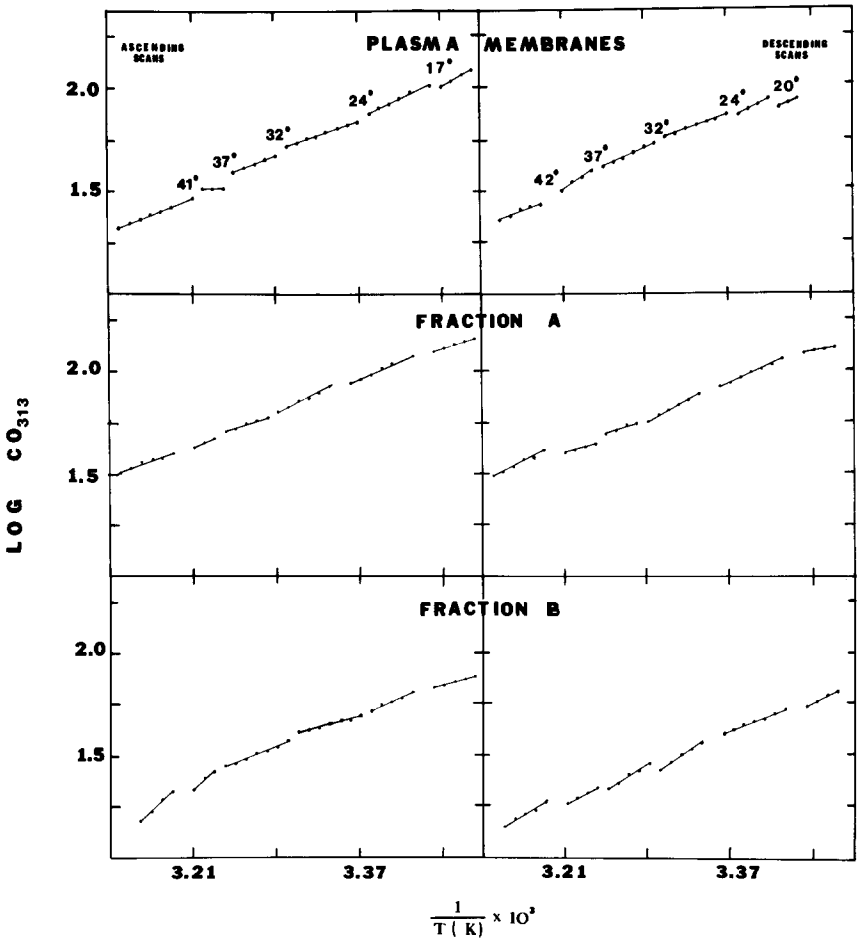
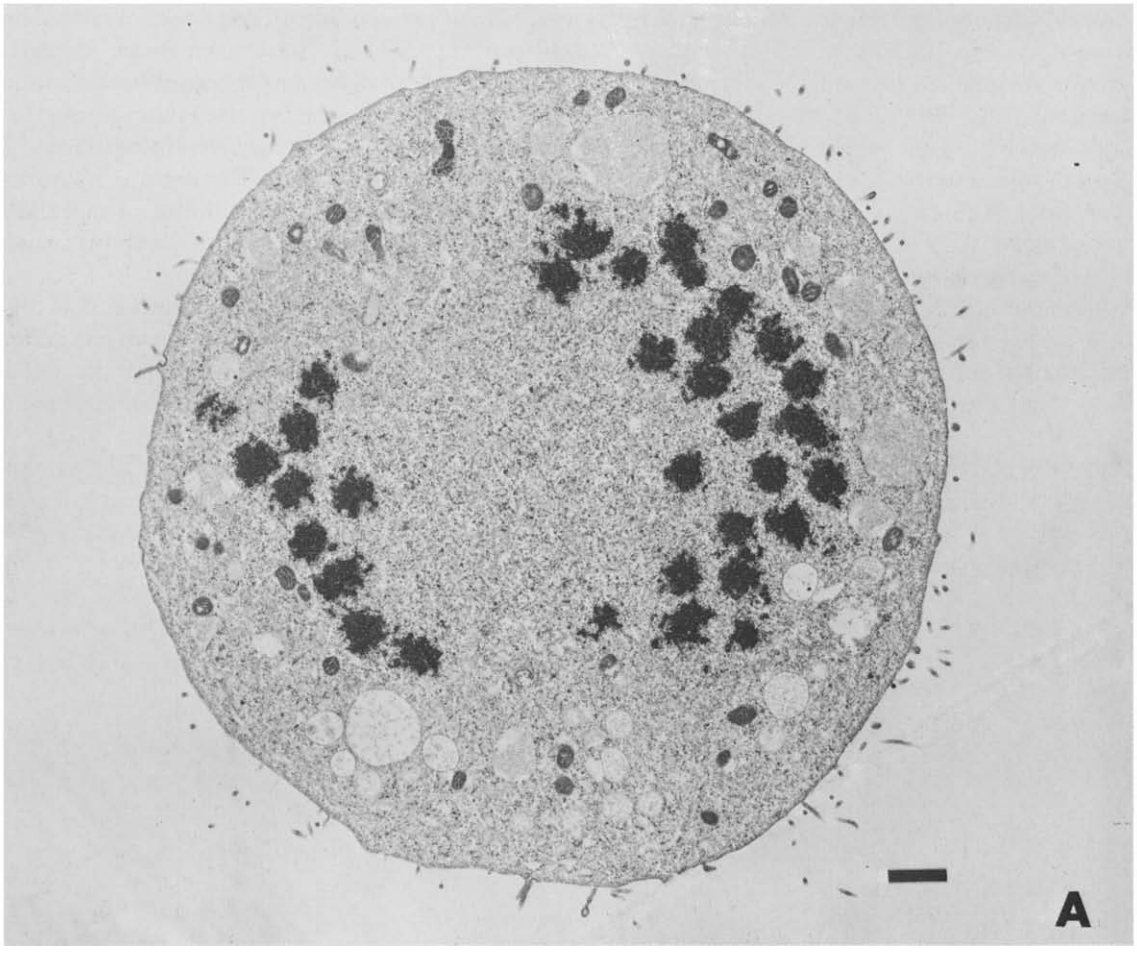
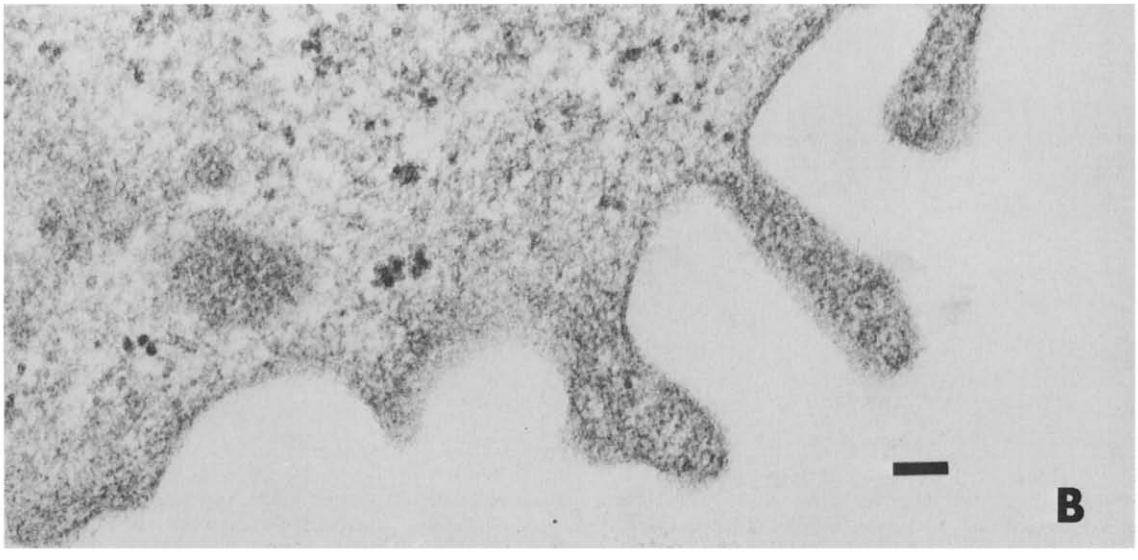


Fig. 3. Arrhenius plots of *trans*-parinaric acid absorbance-corrected fluorescence in LM plasma membrane subfractions. Plasma membrane fractions were exposed to *trans*-parinaric acid as described in Materials and Methods and absorption corrected fluorescence, F_{co}, was determined at 313 nm. Temperature was varied from 10 to 50°C at 2K/min.


A


B

trans-parinaric acid fluorescence in the plasma membrane subfractions were determined to be similar (Fig. 3).

Discussion

The results presented herein indicate that ConA-Sepharose affinity chromatography of LM plasma membrane vesicles obtained by Dounce homogenization yields sealed vesicles with right-side-out orientation. Two types of vesicles are obtained varying primarily in protein/lipid ratios, in the content of sialic acid/mg protein, in the specific activity of 5′-nucleotidase and ($Na^+ + K^+$)-ATPase, and in [^{3}H]ouabain binding/mg protein. The lipid composition of the two subfractions was quite similar. Such results may be explained by the presence of microvilli in LM fibroblasts. One membrane subfraction may represent microvillus fragments while the other represents less structured regions of the cell surface. Certainly functional data on phagocytosis of latex beads and pinocytosis of horseradish peroxidase [33] indicate that the LM cell must have such cell surface projections since it can internalize an equivalent of its surface membrane approximately every three hours. In addition, electron micrographs indicated that LM cells grown in suspension contained numerous microvilli (Fig. 4). Analysis of the isolated membrane subfractions were not, however, consistent with the above interpretation: (a) the lipid composition of both plasma membrane subfraction A and B did not resemble that of the membrane surrounding phagocytosed beads [33], and (b) electron microscopy of the isolated membrane subfractions revealed no obvious differences. An alternate interpretation of these results is that the heterogeneity was due to cell cycle differences during cell differentiation. In the present investigation all cells were a single cell type and were harvested in log phase growth (established from growth curves as described earlier [19]). The exponentially growing cells may contain some stationary cells since the culture was not synchronized. Such cells could possibly give rise to the membrane subfractions. However, when the experiment was repeated using stationary, plateau phase cells a distribution of membrane vesicles similar to that shown in Fig. 1 was obtained. These findings are consistent with the heterogeneous nature of the LM cell surface, although the origin of the heterogeneity remains elusive. Other investigators using heterogeneous cell types also concluded that Con A-Sepharose chromatography yields membrane subfractions representing different 'patches' or 'membrane domains' [2,3,5,36]. In contrast, our data do not support the interpretation that Con A-Sepharose chromatography resolves plasma membrane vesicles into 'right-side-out' or 'inside-out' fractions [4,37] similar to those separated from erythrocyte ghosts [17,38].

Functional and structural mosaicism appears to be a basic property of mammalian cell surface membranes [36,39]. The lateral distribution of membrane anionic sites, for example, is related to the lung colonising potential of B16 melanoma variants [40] and to the agglutinability of malignant transformed cells [41]. Also, the cell surface has a variety of domains which may function in endocytosis or exocytosis [33]. The mosaicism of the cell surface may be explored by investigation of endocytosis or exocytosis. Membranes of enveloped viruses that bud at the cell surface [42,43], phagocytized latex beads [33,44], or membrane surrounding endocytized parasites [45,46] may represent specialized areas of the primary cell surface membrane. However, it is difficult to determine if differences in membrane structure in these endocytized or exocytized structures reflect a nonrandom distribution of membrane components that provides specialized sites or if these processes themselves induce lateral rearrangements of mem-

Fig. 4. Electron micrograph of LM cells cultured in suspension. LM cells were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer for 1–2 h at 4°C. After several rinses and an overnight wash in the buffer, the specimens were postfixed in buffered 2% OsO_4 for 1–2 h at 4°C. The samples were then rinsed with several changes of buffer, dehydrated in a graded ethanol series (20, 40, 60, 80, 95, and 100%) and embedded in Spurr's low viscosity resin [34]. Ultrathin sections were cut on an LKB ultramicrotome with a diamond knife and were poststained with uranyl acetate and lead citrate [35]. Micrographs were obtained with an RCA EMU-3G electron microscope operating at an accelerating voltage of 100 kV. Bar in A represents 1.0 μm; bar in B represents 0.1 μm.

brane components. The results presented here and by others [2,3,5,36] indicate that Con A-Sepharose may provide an excellent method for separation of heterogeneous plasma membrane vesicles. This is particularly important since certain plasma membrane isolation procedures may select for more rigid fragments [47].

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