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Rapid isolation of neuroblastoma plasma membranes on Percoll gradients. Characterization and lipid composition

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A purified plasma membrane fraction was isolated from cultured neuroblastoma (N1E-115) cells on a discontinuous gradient of 5, 25 and 35% Percoll within 1 h of cell disruption by nitrogen cavitation. Yield of plasma membrane, banding in the 25% Percoll ($d = 1.051$), was high as judged by the recoveries of the marker enzymes, 5′-nucleotidase (58.0 ± 5.4%, $n = 5$), alkaline phosphatase (46.0 ± 3.0%, $n = 4$) and Mg^{2+}-stimulated neutral sphingomyelinase (48.0 ± 4.2%, $n = 3$); enrichment of specific activities of these enzymes relative to total cell homogenate (lysate) were 10.9 ± 1.0-, 9.1 ± 1.0- and 9.6 ± 0.4-fold, respectively. Levels of marker enzymes for other organelles were less than 3% of total activity, except for microsomes (less than 9%). The plasma membrane fraction was further characterized by 2-, 5- and 6-fold higher content (nmol/mg protein) of total phospholipids, free cholesterol and sphingomyelin, respectively, compared to lysate. Ratios of free cholesterol to phospholipids and of sphingomyelin to phosphatidylcholine in the plasma membrane fraction were about 2-fold greater than that of lysate. The cholesterol ester content of plasma membrane (36 ± 8 nmol/mg protein) was 2–3-fold higher than that of lysate. Sphingomyelin of the plasma membrane fraction had a higher concentration of long-chain fatty acids (more than 18 carbon atoms) relative to lysate or microsomes. Significant differences also were observed in the fatty acyl composition of diphosphatidylglycerol, cholesterol esters and triacylglycerol of plasma membrane. Thus, we have devised a rapid and reliable method for isolation of highly purified plasma membranes of cultured neuroblastoma cells that is suitable for comparison of metabolic relationships between the plasma membrane and other cellular organelles.

Introduction

The plasma membrane behaves as a dynamic organelle rather than as a passive sieve or static border [1,2]. A unique lipid composition contributes to the specialized and dynamic properties of the plasma membrane: compared to endoplasmic reticulum or mitochondria, plasma membranes are enriched in sphingomyelin and phosphatidylserine and are characterized by a high cholesterol-to-phospholipid molar ratio [3–5]. Although the fatty acyl chain composition of the phospholipids of plasma membrane does not differ very much from other intracellular membranes, changes in the degree of unsaturation have been observed following exposure of cultured cells to polyenoic fatty acids

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Abbreviation: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

[6], after stimulation with mitogens [7], or in comparisons of normal liver and hepatoma cells [8].

Although various hypothetical models of plasma membrane generation and its relationship to intracellular membranes have been postulated (e.g., Refs. 9,10), the contribution of fatty acid composition and turnover of membrane lipids to this process is not well understood. In preliminary studies of labeled fatty acyl chain incorporation into cultured neuroblastoma cells, using our previous method for isolation of plasma membranes from this cell line [11], we observed incorporation and turnover of fatty acyl chains in plasma membrane phospholipids. To study this process in greater detail, we required a more rapid method for the isolation of multiple samples of plasma membrane with relatively high yield and purity.

Several investigators have used Percoll for separation of intact cells and subcellular fractions [12–15]. We herein report a rapid and simplified method for isolation of plasma membranes from cultured neuroblastoma cells on a discontinuous Percoll gradient at alkaline pH. We have carried out a detailed enzymatic and chemical characterization of isolated plasma membranes and other cellular fractions. The chemical characterization includes the first report of the complete fatty acyl chain composition of the plasma membranes and microsomal fractions of the N1E-115 neuroblastoma cell line widely used in a variety of cultured-cell studies.

Materials and Methods

Materials. Chemicals and reagents used were of the finest quality available and were purchased from various commercial suppliers. Organic solvents (HPLC grade) and precoated silica-gel G thin-layer chromatography plates were from Fisher Scientific, Dartmouth, NS. Percoll was from Pharmacia Fine Chemicals, Montreal, PQ. Substrates for enzyme analyses were from Sigma Chemical Co., St. Louis, MO, and [^{3}H(G)]adenosine 5′-monophosphate (15 Ci/mmol) and uridine diphospho[U-^{14}C]galactose (300 mCi/mmol) were from New England Nuclear-Canada, Lachine, PQ. [*methyl*-^{3}H]Sphingomyelin was synthesized [16] in our laboratory. Lipid standards were purchased from Supelco Inc., Bellefonte, PA, and Serdary Research Laboratory, Inc., St. Catherines, ON.

Cell culture. Murine neuroblastoma cells (N1E-115) were cultured in 150 cm^2 flasks in medium containing 5% fetal calf serum and 5% newborn calf serum, as described elsewhere [17]. 7–8 days following subculture, the medium was replaced with 15 ml Dulbecco's minimal essential medium without serum for 24 h and then the cells were harvested by scraping with a rubber policeman.

Isolation of membranes. All operations were at 0–4°C. Cells were washed once with phosphate-buffered saline, (35–45) · 10^6 cells were suspended in 5 ml 0.25 M sucrose/0.2 mM EDTA (lysis medium), equilibrated with nitrogen in a Kontes pressure homogenizer (Kontes, Vineland, NJ) at 100 lb/in^2 for 3 min and released dropwise. The resulting cell lysate was spun at 1000 × *g* for 5 min, the pellet was resuspended in 3 ml fresh lysis medium, and sedimented again as above. The resulting pellet was designated as the crude nuclear fraction. After adjusting to 2.5 mM $MgCl_2$, 3 ml of the combined supernatant (postnuclear supernatant) was gently layered on a discontinuous Percoll gradient.

Stock Percoll (100%) was diluted to give 5, 25 and 35% Percoll solutions in 0.25 M sucrose/50 mM Tricine/2.5 mM $MgCl_2$ (pH 8.8) and 3, 3 and 4 ml, respectively, of these solutions were layered into a 15-ml glass centrifuge tube (18 × 100 mm) using a graduated syringe. The gradient was spun at 27 000 × *g* for 30 min, and five gradient fractions (A_1–A_5; 3, 3, 3, 3 and 2 ml, respectively) were collected from the top of the gradient using pipette.

For preparation of the microsomal fraction, a 3-ml aliquot of postnuclear supernatant was centrifuged at 9750 × *g* for 10 min. The pellet was resuspended in 1 ml lysis medium and centrifuged again. The combined supernatant was adjusted to 1 mM $MgCl_2$, layered on an identical Percoll gradient, centrifuged and fractions (A'_1–A'_5) were collected as described above.

Analytical methods. The marker enzymes were assayed as previously described [11]. Additional enzymes measured were alkaline phosphatase (EC 3.1.3.1, Ref. 18), UDPgalactose-*N*-acetylglucosamine galactosyltransferase [19], succinate dehydrogenase (EC 1.3.99.1, Ref. 20) and lactate dehydrogenase (EC 1.1.1.27, Ref. 21).

Protein was measured fluorometrically [22] with bovine serum albumin as standard.

Lipid analyses. Total lipids were extracted [23], and neutral lipids and phospholipids were separated by silicic acid column and thin-layer chromatography (TLC) [24]. The solvent system used for resolution of phospholipids was chloroform/ethanol/water/triethylamine (4 : 5 : 1 : 4, v/v). Phospholipid phosphorous was quantitated by the method of Fiske and SubbaRow [25]. Cholesterol was measured using a cholesterol oxidase method [26,27] with slight modifications. Total cholesterol was assayed by coupling cholesterol esterase and cholesterol oxidase reactions. Free cholesterol was measured in the presence of cholesterol oxidase only, and cholesterol ester was calculated by difference. Standard curves with purified cholesterol and cholesterol oleate were unaltered by addition of phospholipids or neutral lipids at levels comparable to those in the membrane fractions.

The fatty acyl chain composition of the lipid classes was analyzed by gas-liquid chromatography (GLC) with a 17 : 0 internal standard. Fatty acyl methyl esters [28] were analyzed on a Hewlett-Packard model 5710A gas chromatograph equipped with flame ionization detectors. A 1.7 m × 2 mm (internal diameter) glass column packed with 15% Silar 10 C on Gaschrom R was run isothermally at 195°C with a N_2 carrier flow-rate of 20 ml/min. The integrator (Hewlett-Packard Model 3390A) was programmed to quantitate fatty acyl chains in the samples using relative response factors for each fatty acid and comparison to the internal standard.

Statistical analyses were done using Student's *t*-test.

Results

Cell lysis

Conditions for cell disruption were critically evaluated to minimize sedimentation of plasma membranes in the nuclear fraction. Record et al. [12] observed for Krebs II Ascite cells that the loss could be reduced by including $MgCl_2$ and ATP in the lysis medium and by raising the pH to 9.6, and other workers have used buffered medium containing different salts for cell lysis [29]. However, we found that use of $MgCl_2$ and/or Tricine or borate buffer at several different concentrations resulted in sedimentation of 30–40% of plasma membrane markers with the crude nuclear fraction. Our final procedure was to disrupt the cells in 0.25 M sucrose/0.2 mM EDTA by nitrogen cavitation (100 lb/in^2, 3 min), which resulted in disruption of more than 90% of the cells, as judged by phase contrast microscopy and less than 10% loss of membrane marker enzymes to the nuclear fraction (Table I).

Membrane isolation

After several attempts with limited success to use self-generating gradients of Percoll, we developed a method for plasma membrane isolation on a discontinuous Percoll gradient. As demonstrated previously [12], we observed that at pH 7.5–8.0 the resolution of plasma membrane was poor with heavy contamination by the microsomal fraction. However, good resolution was achieved at pH 8.8. Of several $MgCl_2$ concentrations (0–20 mM) used in the gradient, 2.5 mM $MgCl_2$ gave the highest yield and purity of marker enzymes in the plasma membrane fraction. At other concentrations of $MgCl_2$, plasma membranes remained associated with the bottom fraction (fraction A5). For isolation of the microsomal fraction, centrifugation of the postnuclear supernatant was included to sediment mitochondrial and lysosomal fractions.

Distribution of marker enzymes

The plasma membrane markers, 5′-nucleotidase, alkaline phosphatase and Mg^{2+}-stimulated neutral sphingomyelinase (shown to be preferentially located in plasma membranes of neuroblastoma cells, Ref. 11) were enriched in fraction A_3 of the gradient both in yield and relative specific activity (Table I). Yield of protein was 5–6% of lysate. Marker enzymes for other subcellular organelles were studied to determine the purity of plasma membrane. Activity of hexosaminidase (lysosomes), UDPgalactosyltransferase (Golgi), succinate dehydrogenase (mitochondria) and lactate dehydrogenase (cytosol) was minimal, with approx. 1–5% of the total cell activity being recovered in fraction A_3. NADPH-cytochrome *c* reductase (an endoplasmic reticulum marker) was a persistent minor component of the plasma membrane fraction.

TABLE I

DISTRIBUTION OF PROTEIN AND MARKER ENZYME ACTIVITIES IN SUBCELLULAR FRACTIONS OF NEUROBLASTOMA CELLS

Activities in subcellular fractions are expressed as percent of total cell lysate activity. Enrichment factors (relative specific activities, given in parentheses) were obtained by dividing the specific activity of each fraction by the specific activity of cell lysate. Results are expressed as mean ± S.D. Specific activities of enzymes in the total cell lysate, expressed as nmol/min per mg protein, and the number (n) of subcellular preparations were: 5′-nucleotidase, 5.3 ± 1.2 (n = 5); alkaline phosphatase, 100.0 ± 18.5 (n = 4); Mg^{2+}-stimulated neutral sphingomyelinase, 0.6 ± 0.1 (n = 3); succinate dehydrogenase, 26.4 (n = 2); hexosaminidase, 16.6 ± 2.8 (n = 5); UDPgalactosyl transferase, 16.1 (n = 2); lactate dehydrogenase, 0.9 ± 0.1 units/min per mg (n = 3); and NADPH-cytochrome c reductase, 7.4 ± 1.0 units/min per mg (n = 5).

Constituent	Nuclear fraction	Gradient fractions (A)					Recovery (%)
		1	2	3	4	5	
Protein	13.3 ± 1.9	41.5 ± 3.1	8.8 ± 0.6	5.2 ± 0.3	3.3 ± 0.6	14.5 ± 2.4	86.6 ± 6.3
5′-Nucleotidase	4.7 ± 1.5 (0.4 ± 0.1)	5.2 ± 1.7 (0.1 ± 0.0)	15.8 ± 3.0 (1.8 ± 0.4)	57.8 ± 5.4 (10.9 ± 1.0)	9.4 ± 0.6 (2.9 ± 0.5)	18.2 ± 4.0 (1.3 ± 0.5)	110.1 ± 9.4
Alkaline phosphatase	5.1 ± 1.1 (0.3 ± 0.1)	12.8 ± 1.4 (0.3 ± 0.1)	11.8 ± 2.5 (1.3 ± 0.4)	46.2 ± 2.9 (9.1 ± 1.0)	6.0 ± 1.9 (2.1 ± 0.8)	10.7 ± 4.1 (0.8 ± 0.2)	92.6 ± 5.2
Sphingomyelinase	9.1 ± 0.6 (0.6 ± 0.1)	8.9 ± 0.6 (0.2 ± 0.0)	11.2 ± 1.3 (1.3 ± 0.2)	48.4 ± 4.3 (9.6 ± 0.4)	8.3 ± 0.9 (2.7 ± 0.9)	23.9 ± 0.6 (2.1 ± 0.4)	109.8 ± 6.5
Succinate dehydrogenase	15.0 (1.1)	2.9 (0.1)	1.2 (0.1)	1.5 (0.3)	5.3 (1.8)	67.7 (4.5)	93.6
Hexosaminidase	16.4 ± 3.5 (1.2 ± 0.1)	22.1 ± 4.6 (0.5 ± 0.1)	4.6 ± 1.1 (0.5 ± 0.1)	4.8 ± 0.6 (0.9 ± 0.1)	7.0 ± 1.8 (2.1 ± 0.5)	40.3 ± 11.8 (2.8 ± 0.6)	95.2 ± 14.0
UDPgalactosyl transferase	5.1 (0.4)	17.2 (0.4)	14.1 (1.7)	3.2 (0.6)	10.8 (2.3)	37.5 (2.9)	87.9
Lactate dehydrogenase	4.6 ± 0.8 (0.3 ± 0.1)	56.6 ± 3.7 (1.4 ± 0.0)	12.7 ± 1.1 (1.5 ± 0.1)	2.8 ± 0.5 (0.6 ± 0.1)	0.7 ± 0.3 (0.2 ± 0.1)	1.3 ± 1.0 (0.1 ± 0.1)	78.7 ± 4.3
NADPH-cytochrome c reductase	15.4 ± 2.0 (1.1 ± 0.2)	17.7 ± 5.2 (0.4 ± 0.1)	8.0 ± 1.8 (0.9 ± 0.3)	7.6 ± 1.2 (1.4 ± 0.3)	8.2 ± 1.2 (2.5 ± 0.3)	35.6 ± 5.7 (1.7 ± 0.8)	92.5 ± 6.7

Fraction A'_5 of the gradient was characterized by a high content and relative specific activity of NADPH-cytochrome c reductase compared to total cell lysate (Table II). The contribution of other marker enzymes in that fraction varied from 1 to 10% of their lysate activity.

Lipid composition of membrane fractions

The phospholipid content per mg protein of plasma membrane was 2.3-fold higher than that of lysate (Table III), in agreement with that reported by Charalampous [5] for neuroblastoma cells. The phospholipid content of our plasma membrane fraction did not differ significantly from that of microsomes on a per mg protein basis, whereas Charalampous [5] found that the phospholipid content of microsomes was 1.5-fold higher than that of plasma membranes. The general pattern of phospholipid values obtained by the two methods used by us were in close agreement, although greater deviation from the mean and slightly lower (10–20%) values were observed from GLC analysis.

The 4–5-fold enrichment of cholesterol per mg protein in plasma membrane relative to that of lysate (Table III) was similar to that previously reported [5] although the actual cholesterol content in our cells and membrane fractions was 1.5–2-fold higher. The cholesterol-to-phospholipid ratio of the plasma membrane fraction was about 2-fold higher than that of lysate and was similar to that reported by Charalampous [5]. Cholesterol ester per mg protein in plasma membrane also was 2–3-fold higher than in the lysate. In addition to quantitation by difference in the esterase-oxidase assay, cholesterol ester was quantitated by GLC analysis of the fatty acyl chains following transmethylation and by subjecting the cholesterol ester fraction separated on TLC to chemical hydrolysis and cholesterol oxidase assay. Values obtained by

TABLE II

RECOVERY AND RELATIVE SPECIFIC ACTIVITY OF MARKER ENZYMES IN THE MICROSOMAL FRACTION OF NEUROBLASTOMA CELLS

Activities of each enzyme in fraction A'_5 were compared to that of total cell lysate. Results are expressed as mean percent recovered ± S.D. and as relative specific activity ± S.D. (see Table I for further details).

Enzyme	% Total activity	Relative specific activity
NADPH-cytochrome *c* reductase	25.2 ± 2.0	4.2 ± 0.4
5'-Nucleotidase	8.1 ± 2.4	1.5 ± 0.4
Alkaline phosphatase	3.7 ± 1.4	0.8 ± 0.2
Sphingomyelinase	6.6 ± 3.6	1.6 ± 0.9
Lactate dehydrogenase	0.8 ± 0.4	0.2 ± 0.1
Succinate dehydrogenase	4.4	1.0
Hexosaminidase	7.5 ± 0.8	1.5 ± 0.3
UDPgalactosyl transferase	8.4	1.7
Protein	5.2 ± 0.7	

all three methods were in close agreement, confirming the identity of cholesterol ester.

Generally, there were no significant differences in phospholipid distribution between subcellular fractions except for sphingomyelin, which showed 2-fold enrichment in plasma membrane compared to cell lysate (Table IV). Actual content of sphingomyelin (per mg protein) was about 6-fold higher in plasma membranes (35 nmol) compared to that of lysate (5.8 nmol). As is characteristic of plasma membrane [30], the sphingomyelin-to-phosphatidylcholine ratio was 2-fold higher in plasma membrane compared to lysate. We found a slightly lower phosphatidylserine content in all cell fractions and higher phosphatidylethanolamine content in lysate compared to results reported by Charalampous [5]. Variable amounts of diphosphatidylglycerol were detected in all fractions.

Fatty acyl chain composition

The fatty acid compositions of phospholipids of total cell and subcellular fractions did not differ strikingly from each other (Table V). Except for

TABLE III

PHOSPHOLIPID AND CHOLESTEROL CONTENT OF LYSATE, CYTOSOL, PLASMA MEMBRANE AND MICROSOMES OF NEUROBLASTOMA CELLS

A comparison of two different methods for determining phospholipid content and of the values obtained by other authors for the lipid content of subcellular fractions from these cells. P assay, phospholipid phosphorus assay.

Cell fraction	Phospholipids			Cholesterol		Ratio of free cholesterol to phospholipid
	P assay ($n = 11$)	GLC assay [a] ($n = 4$)	ratio of P to GLC assays ($n = 3$)	free ($n = 5$)	ester ($n = 5$)	
Lysate	180 ± 9 [b] (149 [c], 170 [d])	154 ± 19	1.2 ± 0.1	55 ± 8	14 ± 4 (33 [c], 45 [e]) [f]	0.33 ± 0.06 (0.22 [c], 0.36 [e]) [f]
Cytosol	51 ± 9	41 ± 10	1.1 ± 0.1	18 ± 3	7 ± 3	0.39 ± 0.09
Plasma membrane	412 ± 55 (340 [c])	401 ± 72	1.2 ± 0.0	265 ± 44	38 ± 6 (177 [c]) [f]	0.63 ± 0.14 (0.52 [c]) [f]
Microsomes	385 ± 40 (520 [c])	368 ± 93	1.1 ± 0.0	90 ± 13	42 ± 11 (62 [c]) [f]	0.25 ± 0.08 (0.12 [c]) [f]

[a] Phospholipids were quantitated assuming 2 moles fatty acid per mole phospholipid. (For sphingomyelin, lysophospholipids and cholesterol ester, a mole-to-mole ratio was used.)

[b] Values are nmol/mg protein; mean ± S.D. for the number of isolations of the fractions given in parentheses.

[c] From Charalampous [5].

[d] From Montaudon et al. [36].

[e] From Robert et al. [35].

[f] Total cholesterol (free + esterified).

TABLE IV

DISTRIBUTION OF INDIVIDUAL PHOSPHOLIPIDS IN LYSATE, PLASMA MEMBRANE, MICROSOMES AND CYTOSOL OF NEUROBLASTOMA CELLS AND A COMPARISON WITH VALUES OBTAINED BY OTHER INVESTIGATORS

Individual phospholipids were separated on TLC and quantitated using the phospholipid phosphorus assay as described in Materials and Methods. LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine and ethanolamine plasmalogen; DPG, diphosphatidylglycerol; n.d., not detected.

Lipid fraction or ratio	Lysate	Plasma membrane	Microsomes	Cytosol
LPC	1.5 ±1.6 [a]	1.5 ±0.9 (0.6 [b])	n.d.	1.8 ±2.3
SM	3.6 ±0.1 (2.0 [b], 3.2 [c])	7.9 ±1.3 (8.2 [b])	3.7 ±1.1 (11.0 [b])	3.8 ±0.9
PC	50.2 ±3.1 (57.1 [b], 54.1 [c])	49.3 ±1.9 (40.6 [b])	56.6 ±5.6 (42.9 [b])	53.0 ±3.6
PS	5.1 ±1.7 (15.4 [b] 4.4 [c])	4.3 ±0.9 (14.1 [b])	5.4 ±1.1 (11.9 [b])	4.8 ±2.6
PI	9.4 ±1.6 (10.7 [b], 10.7 [c])	9.9 ±2.0 (6.7 [b])	9.8 ±2.9 (5.0 [b])	10.8 ±1.8
PE	26.1 ±0.3 (12.8 [b], 27.8 [c])	23.5 ±2.9 (30.6 [b])	20.1 ±4.0 (28.0 [b])	21.4± 4.0
DPG	4.6 ±1.6	3.4 ±2.4	5.2 ±2.2	5.1 ±1.4
SM/PC	0.07±0.00 (0.04 [b], 0.06 [c])	0.16±0.02 (0.20 [b])	0.07±0.03 (0.26 [b])	0.06±0.01

[a] Values are the mean ± S.D. for the analyses carried out on four separate isolations of the fractions and are expressed as % of total phosphorus recovered. Recovery of phosphorus from the plate was between 90 and 100%.

[b] Values from Charalampous [5].

[c] From Robert et al. [35].

TABLE V

DISTRIBUTION OF FATTY ACYL CHAINS IN THE TOTAL PHOSPHOLIPID OF TOTAL CELL AND SUBCELLULAR FRACTIONS OF NEUROBLASTOMA CELLS

Total phospholipid was transesterified and the fatty acid methyl esters were analyzed by GLC as described in Materials and Methods. Results are the mean ± S.D. of five independent analyses and are expressed as mol% of the total phospholipid fatty acyl chains. n.d., not detected.

Fatty acyl chains	Lysate	Plasma membrane	Microsomes	Cytosol
14:0	6.4±3.0	3.9±1.8	5.2±3.7	4.5±1.8
16:0	16.7±2.0	18.2±4.2	19.4±2.7	13.0±1.3
16:1	6.9±0.4	7.7±1.5	8.2±1.6	5.4±1.6
18:0	12.8±2.1	14.1±1.4	12.7±1.1	14.0±2.1
18:1	34.4±1.6	35.6±4.9	38.4±4.0	39.2±3.8
18:2 ($n-6$)	5.6±1.0	5.5±0.7	4.8±0.7	5.4±0.8
18:3 ($n-6$)	4.0±0.9	3.8±0.7	3.7±0.9	4.6±1.4
18:3 ($n-3$)	1.5±0.5	1.6±0.7	1.2±0.4	1.2±0.3
20:0	0.6±0.4	0.8±0.4	0.5±0.3	0.6±0.3
20:3 ($n-6$)	1.6±0.2	1.8±0.8	1.3±0.5	1.5±0.3
20:4 ($n-6$)	6.3±0.9	5.9±0.8	4.5±0.8	6.8±1.1
22:5 ($n-3$)	1.2±0.6	1.8±0.5	n.d.	1.6±0.4
24:0	1.3±0.9	0.5±0.3	0.4±0.4	1.6±1.4

TABLE VI

DISTRIBUTION OF FATTY ACYL CHAINS IN THE SPHINGOMYELIN OF TOTAL CELL AND SUBCELLULAR FRACTIONS OF NEUROBLASTOMA CELLS

Sphingomyelin was isolated by TLC, transesterified and the fatty acid methyl esters were analyzed by GLC as described in Materials and Methods. Results are the mean ± S.D. of four independent analyses and are expressed as mol% of the total sphingomyelin fatty acyl chains.

Fatty acyl chains	Lysate	Plasma membrane	Microsomes
14:0	5.9 ± 2.8	0.5 ± 0.2	3.1 ± 1.2
16:0	43.0 ± 6.9	23.0 ± 8.8 [a]	33.2 ± 5.0
16:1	8.8 ± 1.4	7.8 ± 1.4	10.2 ± 2.3
18:0	7.0 ± 1.9	10.1 ± 2.4 [b]	10.7 ± 1.0
18:1	8.4 ± 2.3	12.0 ± 2.4 [a]	22.4 ± 5.8
18:2	0.8 ± 0.2	0.8 ± 0.5	1.4 ± 1.0
20:0	5.3 ± 1.6	10.0 ± 3.7 [a]	2.8 ± 1.7
22:0	6.4 ± 2.3	14.4 ± 5.8 [a]	4.4 ± 1.8
24:0	9.2 ± 1.1	11.4 ± 3.3 [c]	6.8 ± 1.8
24:1	6.0 ± 1.4	10.6 ± 0.3 [a]	5.2 ± 1.3

[a] $P < 0.005$, values compared to lysate and microsomes.
[b] $P < 0.005$, values compared to lysate.
[c] $P < 0.005$, values compared to microsomes.

TABLE VII

DISTRIBUTION OF FATTY ACYL CHAINS IN THE PHOSPHATIDYLCHOLINE OF TOTAL CELL AND SUBCELLULAR FRACTIONS OF NEUROBLASTOMA CELLS

Phosphatidylcholine was isolated by TLC, transesterified and the fatty acid methyl esters were analyzed by GLC as described in Materials and Methods. Results are the mean ± S.D. of four independent analyses and are expressed as mol% of the total phosphatidylcholine fatty acyl chains. n.d., not detected.

Fatty acyl chains	Lysate	Plasma membrane	Microsomes
14:0	4.3 ± 0.5	4.6 ± 1.6	5.0 ± 1.6
16:0	23.8 ± 2.3	25.4 ± 1.4	20.8 ± 1.3
16:1	8.5 ± 1.7	8.4 ± 0.8	10.0 ± 1.9
18:0	9.0 ± 1.8	10.2 ± 1.2	7.3 ± 1.6
18:1	39.8 ± 1.7	37.4 ± 4.0	40.9 ± 3.1
18:2 ($n-6$)	4.7 ± 0.7	3.7 ± 0.5	4.2 ± 0.7
18:3 ($n-6$)	4.8 ± 0.8	4.7 ± 0.3	5.1 ± 1.1
18:3 ($n-3$)	0.7 ± 0.2	n.d.	1.1 ± 0.3
20:3 ($n-9$)	n.d.	0.8 ± 0.1	n.d.
20:3 ($n-6$)	0.9 ± 0.3	0.9 ± 0.4	1.0 ± 0.3
20:4 ($n-6$)	2.2 ± 0.9	1.9 ± 0.6	1.7 ± 0.4
22:5 ($n-3$)	0.3 ± 0.1	0.7 ± 0.4	1.2 ± 0.8
22:6 ($n-3$)	n.d.	0.3 ± 0.1	0.5 ± 0.1
24:0	0.4 ± 0.2	0.5 ± 0.3	0.8 ± 0.7
24:1	n.d.	0.3 ± 0.2	0.3 ± 0.3

sphingomyelin (Table VI) and diphosphatidylglycerol (Table VIII), the fatty acid composition of individual phospholipids of different fractions also were similar to each other. The fatty acid analysis for phosphatidylcholine is given as an example (Table VII). For sphingomyelin, 16:0 was considerably decreased in plasma membranes compared to cell lysate and microsomes (Table VI). Whereas the 18:1 content of plasma membrane was slightly higher than that of lysate, it was 50% that of microsomes. The long-chain fatty acids, 20:0, 22:0, 24:0 and 24:1, were significantly higher in plasma membrane compared to the other two fractions.

Diphosphatidylglycerol (Table VIII) of lysate had the highest percentage (14–18%) of 18:2 of any of the phospholipids. The plasma membrane differed from the lysate in having a higher concentration of saturated fatty acids. On the other

TABLE VIII

DISTRIBUTION OF FATTY ACYL CHAINS IN THE DIPHOSPHATIDYLGLYCEROL OF TOTAL CELL AND SUBCELLULAR FRACTIONS OF NEUROBLASTOMA CELLS

Diphosphatidylglycerol was isolated by TLC, transesterified and the fatty acid methyl esters were analyzed by GLC as described in Materials and Methods. Results are the mean ± S.D. of four independent analyses and are expressed as mol% of the total diphosphatidylglycerol fatty acyl chains. n.d., not detected.

Fatty acyl chains	Lysate	Plasma membrane	Microsomes
14:0	5.9 ± 2.4	11.8 ± 3.8 [a]	3.5 ± 3.1
16:0	13.5 ± 1.0	23.2 ± 10.3 [b]	25.1 ± 3.0
16:1	13.6 ± 4.5	10.1 ± 4.3	14.4 ± 4.8
18:0	5.5 ± 1.5	7.4 ± 1.7	8.1 ± 0.1
18:1	31.0 ± 4.6	17.5 ± 2.1 [a]	31.4 ± 9.2
18:2 ($n-6$)	16.8 ± 2.3	9.4 ± 1.4 [b]	7.5 ± 1.4
18:3 ($n-6$)	2.3 ± 0.7	2.9 ± 2.0	2.7 ± 1.9
18:3 ($n-3$)	0.6 ± 0.1	3.0 ± 1.4	0.8 ± 0.1
20:0	1.0 ± 0.8	2.1 ± 1.1	1.0 ± 0.2
20:3 ($n-9$)	n.d.	2.9 ± 1.9	n.d.
20:3 ($n-6$)	2.2 ± 0.6	3.3 ± 1.4	2.7 ± 0.7
20:4 ($n-6$)	2.3 ± 0.9	4.7 ± 2.4	2.9 ± 1.7
22:5 ($n-3$)	1.7 ± 0.5	1.8 ± 1.4	n.d.
22:6 ($n-3$)	1.5 ± 0.3	n.d.	n.d.
24:0	0.8 ± 0.7	n.d.	n.d.
24:1	1.3 ± 0.7	n.d.	nd.

[a,b] Refer to footnotes a and b, respectively, of Table VI.

TABLE IX

DISTRIBUTION OF FATTY ACYL CHAINS IN THE TRIACYLGLYCEROL OF TOTAL CELL AND SUBCELLULAR FRACTIONS OF NEUROBLASTOMA CELLS

Triacylglycerol was isolated by TLC, transesterified and the fatty acid methyl esters were analyzed by GLC as described in Materials and Methods. Results are the mean ± S.D. of four independent analyses and are expressed as mol% of the total triacylglycerol fatty acyl chains. n.d., not detected.

Fatty acyl chains	Lysate	Plasma membrane	Microsomes
14:0	6.8 ± 1.0	10.1 ± 1.8 [a]	10.2 ± 1.2
16:0	24.6 ± 1.9	27.9 ± 3.3	24.5 ± 2.3
16:1	6.8 ± 2.4	14.7 ± 0.5 [a]	17.8 ± 7.2
18:0	9.7 ± 1.4	8.6 ± 0.7	7.5 ± 1.9
18:1	30.5 ± 1.2	25.5 ± 2.0 [a]	25.4 ± 0.8
18:2 ($n-6$)	2.6 ± 0.6	2.6 ± 0.5 [b]	5.8 ± 2.1
18:3 ($n-6$)	8.1 ± 1.9	6.0 ± 2.4	4.5 ± 2.9
20:0	2.8 ± 0.7	2.0 ± 0.2	3.3 ± 0.6
20:3 ($n-6$)	1.6 ± 1.2	n.d.	n.d.
20:4 ($n-6$)	2.0 ± 1.0	1.6 ± 0.3	1.0 ± 0.1
24:0	1.9 ± 0.9	1.0 ± 0.4	n.d.
24:1	2.1 ± 0.8	n.d.	n.d.

[a,b] Refer to footnotes b and c, respectively, of Table VI.

TABLE X

DISTRIBUTION OF FATTY ACYL CHAINS IN THE CHOLESTEROL ESTERS OF TOTAL CELL AND SUBCELLULAR FRACTIONS OF NEUROBLASTOMA CELLS

Cholesterol ester was isolated by TLC, transesterified and the fatty acid methyl esters were analyzed by GLC as described in Materials and Methods. Results are the mean ± S.D. of three independent analyses and are expressed as mol% of the total cholesterol ester fatty acyl chains. n.d., not detected.

Fatty acyl chains	Lysate	Plasma membrane	Microsomes
14:0	5.1 ± 4.1	11.0 ± 4.8	7.1 ± 4.4
16:0	18.0 ± 5.3	20.0 ± 7.0	21.5 ± 7.1
16:1	9.8 ± 3.0	20.6 ± 2.0 [a]	14.5 ± 3.1
18:0	9.2 ± 3.0	8.0 ± 2.2	8.7 ± 1.3
18:1	23.0 ± 2.9	16.5 ± 2.3 [a]	20.5 ± 2.4
18:2 ($n-6$)	2.3 ± 1.3	5.8 ± 0.5 [a]	4.3 ± 3.6
18:3 ($n-6$)	13.7 ± 1.8	6.4 ± 2.3' [a]	8.5 ± 1.6
20:0	6.0 ± 1.6	5.0 ± 1.0	5.1 ± 1.1
20:3 ($n-6$)	4.7 ± 0.9	n.d.	3.6 ± 0.1
20:4 ($n-6$)	5.5 ± 2.3	5.0 ± 3.0	6.4 ± 1.5
24:0	3.3 ± 1.6	2.3 ± 1.2	n.d.

[a] Refer to footnote b of Table VI.

hand, 18:1 and 18:2 were reduced by nearly 50% in plasma membrane relative to cell lysate.

The fatty acid profiles of both triacylglycerol and cholesterol esters exhibited significant differences between the total cell and subcellular membrane fractions. Plasma membrane triacylglycerol (Table IX) had a higher proportion of 14:0, and 16:1 and slightly lower proportion of 18:1 than the cell lysate. It differed from microsomes in having significantly lower proportion of 18:2. The fatty acyl chain composition of plasma membrane cholesterol ester also showed significant variations compared to lysate or microsomes (Table X): levels of 16:1 and 18:2 were higher than that of lysate whereas that of 18:1 and 18:3 ($n-6$) were lower. It differed from that of microsomes in having higher concentration of 16:1 and lower concentration of 18:1.

Discussion

Methods for the isolation of plasma membrane of cultured cells utilizing a variety of separation techniques have been reported [29]. In many cases, the procedures have been lengthy and the yield of plasma membrane is often low. Accordingly, we developed a rapid method of isolation of a plasma membrane fraction with high yield based on classical enzyme markers and with minimal contamination by other subcellular organelles.

Within 1 h of harvesting the neuroblastoma cultures, a plasma membrane fraction was obtained containing 45–60% of the total 5'-nucleotidase, alkaline phosphatase and Mg^{2+}-stimulated neutral sphingomyelinase with relative enrichment of 9–12-fold in specific activity compared to the total cell. In contrast, less than 9% of the total cell activity of the enzyme marker for microsomes and less than 3% of the markers for Golgi, mitochondria, lysosomes and cytosol were present in the plasma membrane fraction. This low cross-contamination and high yield exceeds that in the previously published method for neuroblastoma cells [11] and is highly reproducible.

Several factors account for the success of this procedure. First, the choice of an appropriate lysis medium containing EDTA minimized the sedimentation of plasma membrane markers with the crude nuclear fraction. As a result, more than 90%

of membrane enzymes were in the postnuclear fraction from which the membranes were isolated. A similar observation was made by Record et al. [12] for Krebs II ascite cells, but the buffered solution (pH 9.6) containing ATP and $MgCl_2$ which they used to lyse cells was not an advantage for neuroblastoma cells.

Secondly, the pH and $MgCl_2$ concentration of the Percoll gradient were carefully adjusted to yield highly enriched plasma membrane. The higher pH appeared to prevent clumping of subcellular organelles [12], improving the resolution of membranes on the gradient. Steck et al. [31] have suggested a mechanism whereby the mobility of membrane vesicles of different origin are influenced by the pH and ionic strength of the gradient. Arguments [12] for using a high pH are supported by our findings during the development of our isolation procedure.

A preliminary centrifugation of the postnuclear supernatant substantially reduced the contamination of microsomes by mitochondria and lysosomes. However, there was also considerable pelleting of the plasma membrane so that the supernatant obtained could not be used as a source for single-step isolation of both plasma membranes and microsomes on the same gradient. The resultant microsomal preparation was considerably better than that reported earlier [11] both in yield and purity.

The higher content of phospholipids, cholesterol and sphingomyelin also confirm the identity of our isolated plasma membrane. Recoveries of these lipids in the plasma membrane fraction were 14.6 + 2.7% ($n = 6$), 32.2 + 1.9% ($n = 5$), 35.3 + 1.2% ($n = 4$) of their respective amounts in the total cell.

If the assumption is made that the plasma membrane marker enzymes are exclusively located in the plasma membrane, approx. 50% of the total plasma membrane was recovered in our fraction. Based on this, it can be calculated that 25–35% of total phospholipids, 60–70% of total free cholesterol and 70–75% of total sphingomyelin are located in plasma membranes of neuroblastoma cells. Even though this calculation does not take the influence of minor contamination into consideration, the relative amounts of lipid in our plasma membrane fraction are comparable to other reports. For example, the plasma membrane of erythroleukemic cells contain 64.1 ± 6% and 71% of total cholesterol and sphingomyelin, respectively [32]. The relative proportion of total phospholipid in our plasma membrane preparation is less than that reported for erythroleukemic plasma membranes [32] or Krebs II ascite plasma membranes [12].

The cholesterol ester in membrane fractions has not been noted in previous reports of neuroblastoma membrane lipid composition. We used three independent methods to quantitate the cholesterol ester in our fractions and found close agreement in each case. Of the total cholesterol, cholesterol ester accounted for 15–19% in the total cell, 10–17% in the plasma membrane fraction and 40–50% in the microsomal fraction.

It has been generally assumed that cholesterol ester contributes little to total cholesterol content, and no distinction has been made between free and esterified cholesterol during cholesterol estimations. However, Van Hoeven and Emmelot [33] observed that the cholesterol ester content of rat and mouse hepatoma plasma membranes was from 3- to 20-fold higher (on a protein basis) compared to the plasma membrane of normal liver cells. Wood [34] also reported a high content of cholesterol ester in Ehrlich ascites plasma membranes: of the total membrane lipids, about 5% was contributed by cholesterol ester compared to 9–12% in whole cells. It was concluded that the cholesterol ester concentration was significantly higher in tumor cells compared to normal cells. As our neuroblastoma line is of tumour origin, the high cholesterol ester content may be a reflection of the neoplastic nature of the cells.

Although the fatty acid composition of the phospholipids of neuroblastoma cells has been reported [35,36], this is the first report on the acyl chain composition of the phospholipids of neuroblastoma subcellular membranes. Significantly higher levels of ($n - 6$) and relatively lower levels of ($n - 3$) polyunsaturated fatty acids were present in our cell phospholipids as compared to other studies [35,36]. This can be attributed primarily to our use of a mixture of 5% fetal calf serum and 5% newborn calf serum for cell culture, rather than 10% fetal calf serum [35,36]. The fatty acyl chain composition of our fetal calf serum (data not shown) was similar to that reported in the previous

studies [37] except for slightly higher 22 : 5 ($n - 3$) and 22 : 6 ($n - 3$) in their analyses. In contrast to the fetal calf serum, the newborn calf serum had a 2-fold excess of total fatty acyl chains (nmol/ml), a 3-fold greater amount of 18 : 2 ($n - 6$), and only a trace of 22 : 5 ($n - 3$) and 22 : 6 ($n - 3$). Thus, our cells were exposed to more 18 : 2 ($n - 6$) and less 22 : 5 ($n - 3$) and 22 : 6 ($n - 3$) than in the other studies.

Our previous studies [38] and others [35] have shown that neuroblastoma cells readily utilize fatty acids from the medium and incorporate them into complex lipids. Also, Tahin et al. [39] observed that dietary increase of 18 : 2 ($n - 6$) fatty acid not only increased the ($n - 6$) fatty acids of cellular lipids but also suppressed the incorporation of ($n - 3$) fatty acids into membrane lipids. Thus, such incorporation, selectivity and competition for fatty acyl chains available from the medium could account for the relatively low concentration of ($n - 3$) fatty acids in our cell membranes.

The fatty acid profiles of individual phospholipids differed significantly from each other. Lysophosphatidylcholine and sphingomyelin were rich in saturated fatty acids (60–80%) compared to other phospholipids, whereas phosphatidylinositol was enriched in polyunsaturated acyl chains (30–45%), with lesser amounts in phosphatidylethanolamine (25–35%), phosphatidylserine (15–30%) and phosphatidylcholine (10–15%). An opposite trend was seen in the monounsaturated acids (mainly 18 : 1), with highest levels (45–50%) in phosphatidylcholine and decreasing amounts in phosphatidylserine (30–40%), phosphatidylethanolamine (35–45%), phosphatidylinositol (20–30%), lysophosphatidylcholine (20–30%) and sphingomyelin (15–30%).

Although the individual complex lipids differed from each other in fatty acid composition, each one differed little from its counterpart in other cellular fractions. Exceptions were sphingomyelin, diphosphatidylglycerol and cholesterol ester. To explain a similar observation in liver cells [40] and Ehrlich ascites cells [41], it was proposed that the distribution of molecular species of most lipids may be random in a given cell type or organ. While our observations tend to support such a hypothesis, some selectivity in acyl chain composition between membrane compartments was evident, as emphasized in the sphingomyelin, diphosphatidylglycerol and cholesterol ester components. Such a distribution of fatty acyl chains clearly indicated that the molecular species of sphingomyelin in the plasma membrane were quite distinct from that of total cells or of microsomal membrane.

The diphosphatidylglycerol in our plasma membrane preparation is of interest. The presence of this lipid, characteristic of mitochondrial membranes, cannot be dismissed as contamination of the plasma membrane fraction by mitochondria as mitochondrial marker contamination was slight and the diphosphatidylglycerol in the plasma membrane was distinct from that of the total cell and microsomal fraction in its acyl chain composition. It seems unlikely, therefore, that redistribution of lipids between membranes during isolation could be responsible for the diphosphatidylglycerol in plasma membrane preparations [42]. Further, this lipid has been detected in the plasma membranes of normal [33] and neoplastic [12,33,34,42] cells and may be characteristic of the latter as its concentration is high in tumor plasma membranes [33,42]. The levels of 18 : 2 in diphosphatidylglycerol were lower in our fractions as compared to other studies [34,42], however, as in these studies there was a relatively lower concentration of 18 : 2 ($n - 6$) in the membranes as compared to whole cells.

Considerable differences in the fatty acid profile of plasma membrane cholesterol ester compared to total cell indicate that the plasma membrane cholesterol ester may be unique to that membrane. Although the fatty acyl composition of cholesterol ester in our cells differed considerably from that of liver cells and Ehrlich cells [34,40], the occurrence of a relatively higher concentration of 16 : 1 in plasma membrane compared to whole cells is consistent in all the three cell types.

Detectable amounts of triacylglycerol and free fatty acids (data not shown) were also observed in our membrane preparations, as has been found by others [43,8,4]. Although the fatty acyl composition of triacylglycerol of the plasma membrane differed from that of whole cell or microsomes, further studies are required to assess whether these neutral lipids are an integral part of the membrane and may play an active role in the functions and

metabolic turnover of the plasma membrane.

In summary, we have described a method for rapid isolation of highly enriched plasma membranes from neuroblastoma cells and have reported a detailed lipid analysis of whole cells and membrane fractions. Such a reliable and relatively facile method provides a sound basis for future studies of phospholipid and fatty acyl chain metabolism at the membrane level.

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