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Tumor Lipids: Characterization of the Lipids Isolated from Membranous Material

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Membranous material was prepared from Ehrlich ascites carcinoma cells by zonal centrifugation. The distribution of fatty acids among the individual neutral lipid and phospholipid classes isolated from the membrane preparation was determined. Survey electron microscopy indicated that the preparations consisted of vesicles of rough surfaced endoplasmic reticulum, lysosomal-like structures, virus-like particles, few intact mitochondria, and unidentified amorphous material, in addition to plasma membranes.

Lipids and proteins accounted for approximately one-third and two-thirds of the membranous material. Phospholipids accounted for two-thirds of the total lipids. The membranous material contained higher levels of free fatty acids, cholesterol, diacyl phosphatidyl choline, diacyl phosphatidyl ethanolamine, and lower levels of glyceryl ether diesters and cholesterol esters than whole cells. The fatty acid composition of all the lipid classes isolated from the membranous material, except phosphatidyl inositol and cholesterol esters, was similar to the fatty acid distribution of the corresponding classes obtained from whole cells. Cholesterol esters contained elevated levels of 16:1 and stearic acid accounted for 88% of phosphatidyl inositol fatty acids.

Phosphatidyl choline and phosphatidyl ethanolamine isolated from the membranous preparations contained 1% or less alkyl acyl phosphatides, and plasmalogens were not detected. The lipids of whole Ehrlich ascites cells have been shown to contain high levels of ether-linked lipids, whereas these data indicate that alkyl and alk-1-enyl glyceryl ethers are virtually absent from membrane preparations of these cells. These results indicate that the ether-linked lipids are not distributed equally among all cell structures.

Evidence suggests that the invasiveness of neoplastic tissue may be related to the surface and biochemical properties of tumor cell membranes (1, 2). Lipids and protein are the major components of membranes, but the detailed characterization of the membrane lipids derived from neoplastic cells has been limited. Wallach and colleagues (3, 4) have recorded the physical properties of intact membranes of Ehrlich ascites carcinoma cells before and after lipid depletion under a variety of conditions.

Neutral lipids and phospholipid classes of Ehrlich ascites cells have been analyzed in detail (5–7). The previous investigations (5–7) provide a basis whereby the structure

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of lipids obtained from cellular organelles can be compared to the whole cell. Lipids isolated from membranous material are characterized in the present study and compared with the lipids of the whole cell. The data show some striking differences between the lipids of the whole cell and the lipids of the membranous material.

EXPERIMENTAL

Isolation of membranous fraction. Ehrlich ascites carcinoma cells were grown in the peritoneal cavity of Swiss white mice (HA/ICR strain). Each of two membranous fractions were prepared from the cells harvested from approximately 50 mice on the 7th and 8th day after the cells had been transplanted. Clumped cells and those contaminated with large numbers of red blood cells were discarded. Harvested cells were maintained in an ice bath until they were used, usually within 2–3 hr. The cells were allowed to swell by suspending in 2 vol of a 3 mM Tris (2-amino, 2-hydroxy-methyl propane-1,3-diol)–3 mM magnesium solution for approximately 3 min, at which time 5–10 vol of 3 mM Tris–3 mM magnesium–1 mM sodium bicarbonate buffer was added and the cells disrupted by homogenization (10 strokes) in a loose-fitting Dounce homogenizer.

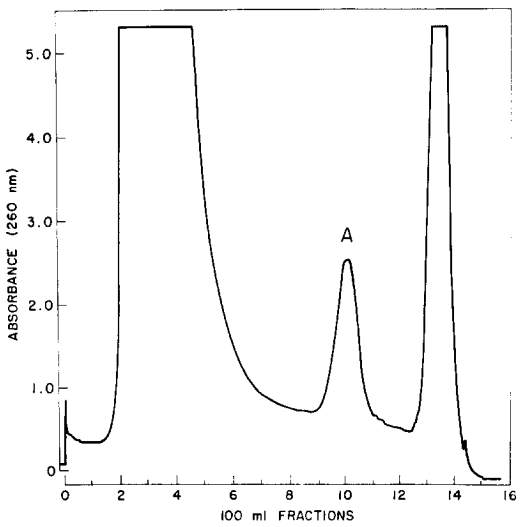

FIG. 1. Absorbance profile of the sucrose gradient containing Ehrlich ascites cell organelles and fragments as unloaded from the zonal rotor after being centrifuged until $w^2t = 2.0 \times 10^8$. The membranous material (peak A) was collected in fractions 9 and 10. Density increased with the increased number of fractions. Details of the sucrose gradient are given in the text.

The membranous fraction was prepared by zonal centrifugation with a B-XV rotor (8). Two hundred milliliters of the homogenate were layered on the light side of a 500-ml linear sucrose gradient (19–35 w/w%). The rotor contained 300 ml of 45% sucrose between the heavy side of the gradient and the 55% sucrose cushion. The sample was overlaid with 200 ml of the Tris–magnesium buffer and centrifuged at 4000 rpm until $w^2t = 2 \times 10^8$. The rotor was unloaded and 100-ml fractions were collected by pumping in 55% sucrose at the outer edge of the rotor. Fractions 9 (39.4% sucrose, 1.179 g/ml) and 10 (46.5% sucrose, 1.217 g/ml) (Fig. 1, Peak A) were diluted 2:1 with 1 mM bicarbonate buffer and the membranous material was sedimented by conventional centrifugation. The supernatant was decanted and the tubes containing the membranous material were stored at −23° until the lipid analyses and fixation for electron microscopy were carried out.

Electron microscopy. An aliquot of the sedimented membranous material obtained from one of two zonal runs was examined by electron microscopy. The frozen fraction was thawed, fixed in 2.5% glutaraldehyde, buffered with 0.1 M sodium cacodylate (pH 7.2) for 30 min, and rinsed overnight in the 0.1 M cacodylate buffer. The sample was postfixed 1 hr in 1% osmium tetraoxide, buffered with 0.1 M cacodylate, and embedded in Epon 812 by the method of Luft (9). Thin sections were cut on an ultramicrotome, picked up on bare copper grids, and double stained in aqueous uranyl acetate and lead citrate (10).

Lipid extraction, fractionation, and analyses. The combined sedimented membranous material from two zonal preparations was diluted with water to a known volume and uniformly dispersed by sonic oscillation for 5 min. Aliquots were taken for protein determination by the Lowry method (11). The membranous fraction was lyophilized and the lipids were extracted by the Bligh and Dyer procedure (12). A second extraction was performed by readjusting chloroform–methanol concentrations to again give a single phase. Each time, the monophase was sonicated.

Because some sucrose was extracted by the latter procedure, the percentage of lipid in the membranous fraction was based upon the amount of lipid soluble in hot chloroform rather than the total weight of material extractable by the Bligh and Dyer procedure. Neutral lipids were separated from the phospholipid fraction by silicic acid chromatography (13). Cholesterol esters, glyceryl ether diesters, triglycerides (TG), free fatty acids, and cholesterol were isolated by thin-layer chromatography (TLC) with a hexane–diethyl ether–acetic acid (80:20:1, v/v) solvent system. Percentages of each neutral lipid class were calculated

from ratios of sample peak (gas-liquid chromatography, GLC) areas to the area obtained for a known quantity of an internal standard added to the sample before esterification or silylation. Cholestane and methyl heptadecanoate served as internal standards for cholesterol and acyl lipids. Percentages of individual phosphatide classes were determined from phosphorous analysis (14) of classes resolved by TLC using a chloroform–methanol–acetic acid–saline (50:25:8:4, v/v) solvent system (15). The fatty acid composition of individual phospholipid classes, isolated by TLC, was determined by GLC. Half of each phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) fractions was reduced with lithium aluminum hydride (16) and the hydrogenolysis products were chromatographed on TLC (diethyl ether–30% ammonium hydroxide, 100:0.5, v/v) to determine the percentage of alkyl and alk-1-enyl glyceryl ethers present in each fraction. The alkyl glyceryl ethers were converted to isopropylidene derivatives (17), methyl docosanoate was added as an internal standard, and the sample analyzed by GLC as described previously (18).

Details of the GLC and TLC operating parameters and conditions have been described (6, 15). Methyl esters of sphingomyelin fatty acids were prepared by the method of Morrison and Smith (19). All other lipid classes were converted to methyl esters by refluxing the sample for 2 hr with a large excess of 2% sulfuric acid in anhydrous methanol. Peak identities are based upon cochromatography with standards before and after hydrogenation. All percentages represent the mean of two or more analyses on the two pooled preparations of membranous material. The source and grade of solvents, standards, reagents, and supplies used in this investigation have been reported (6, 15).

RESULTS AND DISCUSSION

Purity of membranous fraction. The 260-nm absorbance profile, measured as the sucrose gradient containing Ehrlich ascites cell fragments and organelles was unloaded from the zonal rotor, is shown in Fig. 1. The membranous material used in this study was banded in the sucrose gradient at 1.18–1.22 g/ml (Peak A, Fig. 1). Plasma membranes of rat liver have been shown to band sharply at the lower density (20–22). Wallach and Kamat (23) have reported the isolation of membranous material from Ehrlich ascites cells in a sucrose gradient at a lower buoyant density than that reported here; however, they pointed out that nitrogen gas used to rupture the cell broke the plasma membrane into small fragments.

Electron micrographs disclosed few intact mitochondria and showed that the membranous fraction contained a variety of other cell components. Figures 2 and 3A show that the preparations contain small membranous vesicles, fragments of endoplasmic reticulum, and amorphous material of unknown origin, in addition to plasma membranes. Difficulties in obtaining pure plasma membranes from Ehrlich ascites cells have been encountered previously (23–25). The problem centers around the lack of a satisfactory method of disrupting the cell. Disruption of the cell under isoosmotic conditions by rapid decompression of nitrogen gas (25) or by homogenization of cells swollen in a hypertonic solution (these experiments) leads to an apparent formation of small vesicles from plasma membranes. Convolutions and villus-like projections that cover the surface of these neoplastic cells, readily visible from low magnification electron micrographs (26, 27), may contribute to small vesicle formation. Benedetti and Emmelot (28) have suggested that small vesicles observed in isolated plasma membranes from rat liver might represent microvilli of disrupted bile spaces, tubular invaginations of the plasma membrane, or vesicular remnants of smooth endoplasmic reticulum. Figure 3A also shows the presence of lysosomal-like structures that have been characterized in Ehrlich ascites cells by Horvat and colleagues (27, 29). Figure 3B shows a portion of the field under high magnification that contains numerous virus-like particles. Horvat *et al.* (27) have also published electron micrographs of virus-like particles found in the cells of this neoplasm. Although Figs. 2 and 3 show other cell components in the membranous preparations, membranes appear to comprise the major part of the preparation, which makes the comparison of the lipids from the membrane with those of the whole cell worthwhile.

Class composition. Total phospholipids represented two-thirds of the total lipids. A lipid to protein ratio of 0.55 was obtained for the membranous material. This value is slightly higher than those reported for rat

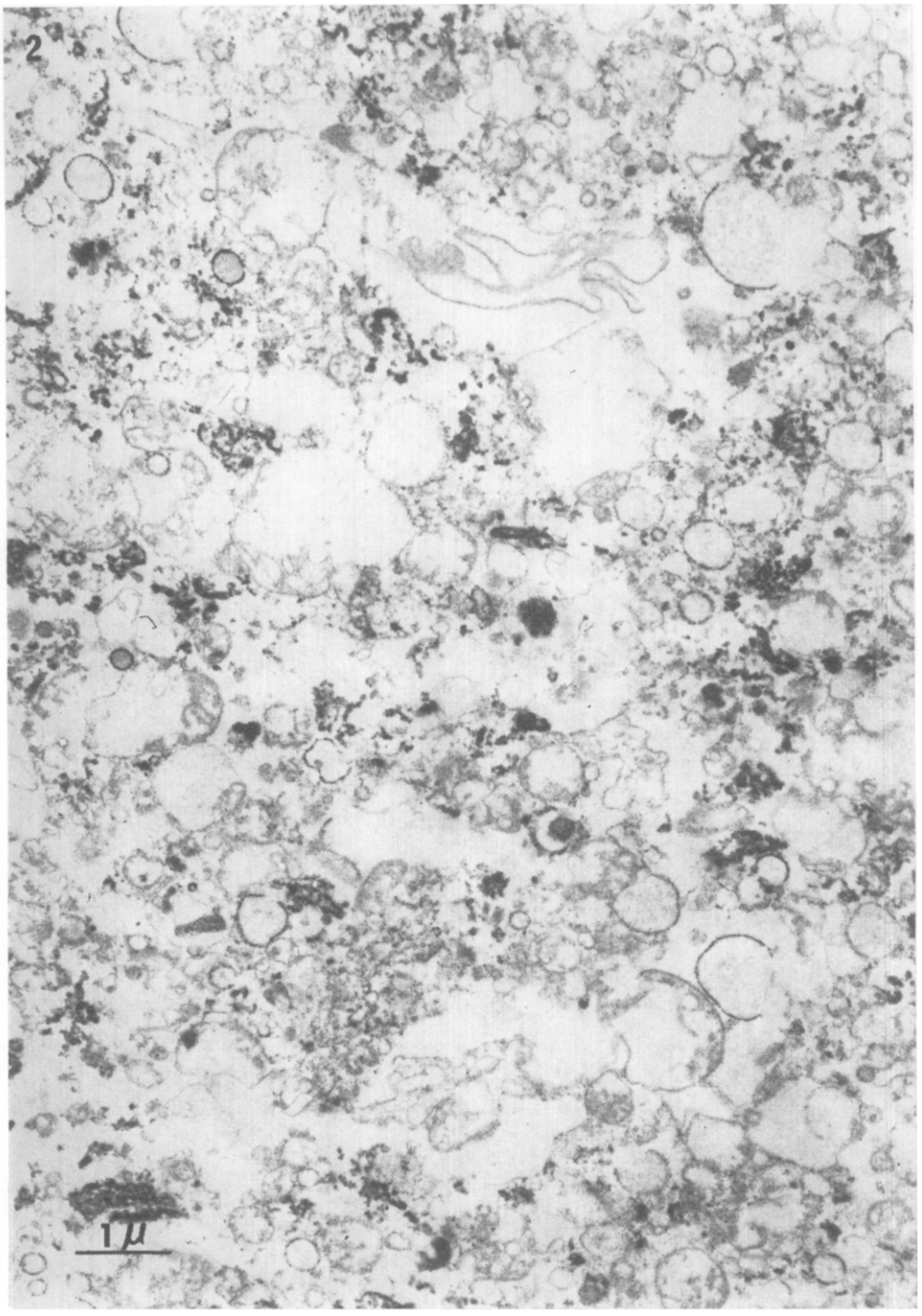

FIG. 2. Survey electron micrograph of membranous material isolated from Ehrlich ascites cells by zonal centrifugation (× 13,500).

liver plasma membranes (21, 30–32). The percentage of each lipid class relative to the total lipid isolated from the membranous material is given in Table I. The lipid class composition of the membranous material was not very different from the percentages Wood and Harlow (7) obtained for the whole cells and those of Lindlar and Wagener

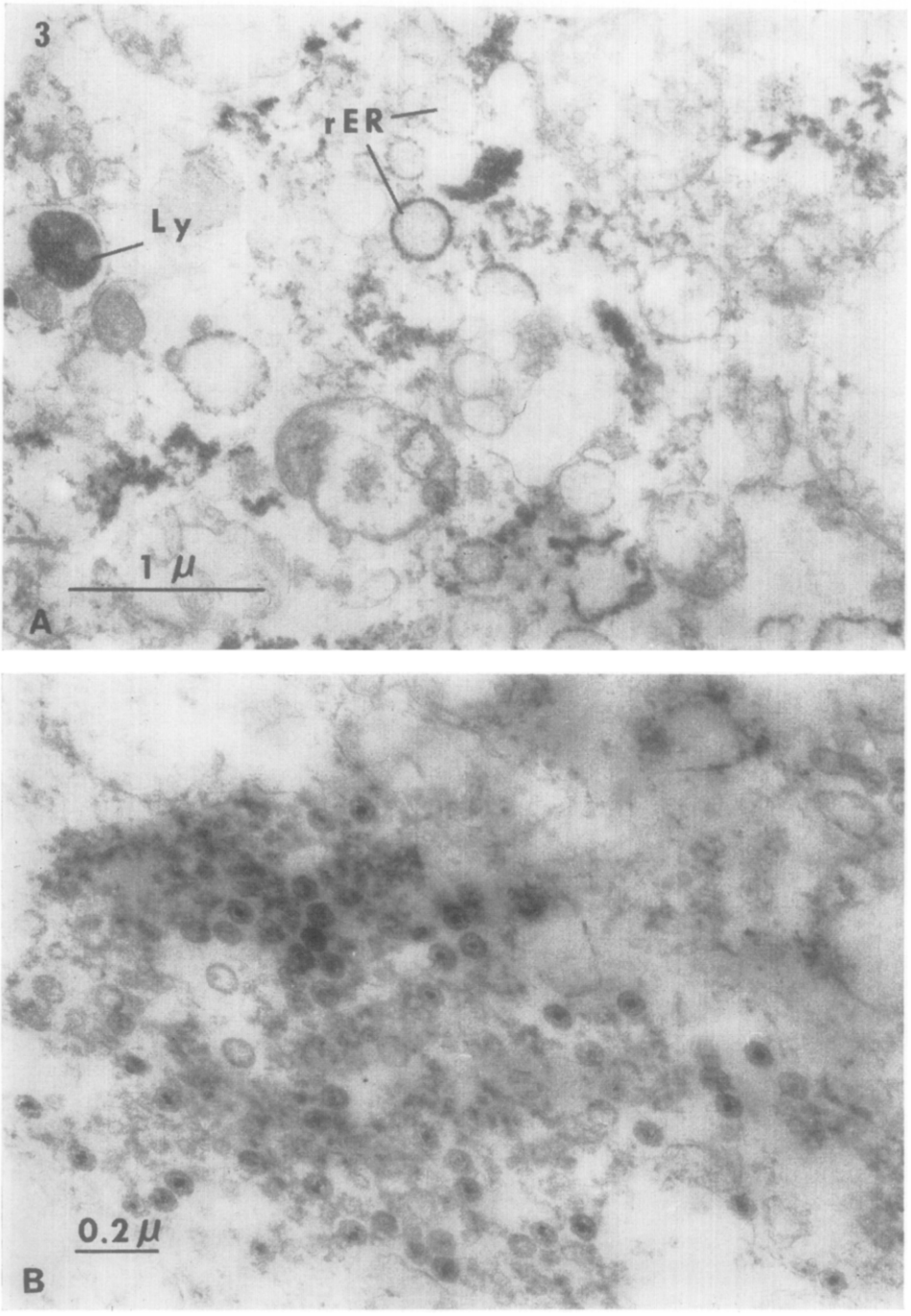

FIG. 3. Electron micrographs of (A) membranous material isolated from Ehrlich ascites cells at higher magnification (× 27,800), and (B) virus-like particles found in the preparation (× 54,000). Vesicles of rough-surfaced endoplasmic reticulum are indicated by rER, and Ly indicates lysosomes.

(33) except for the somewhat higher percentages of lysophosphatidyl choline and free fatty acids we report. Comparable levels of free fatty acids have been reported in the lipids of rat liver plasma membranes (32, 34). Cholesterol ester and glyceryl ether diester percentages were approximately half the values reported for whole cells (5, 34). Cholesterol, the fourth most abundant lipid in the membranous preparations, has been shown to occur at high levels in rat liver plasma membranes (21, 30–32, 34).

TABLE I

LIPID CLASS COMPOSITION OF EHRLICH ASCITES CELL MEMBRANE FRACTION

Lipid class	Percentage[a]
Cholesterol esters	5
Glyceryl ether diesters	1
Triglycerides	10
Free fatty acids	8
Cholesterol	9
Diphosphatidyl glycerol	6
Phosphatidylethanolamine	15
Phosphatidyl serine	2
Phosphatidyl inositol	2
Phosphatidyl choline	29
Sphingomyelin	7
Lysophosphatidyl choline	6

[a] Percentages of individual neutral lipids were calculated from GLC peak areas of the sample relative to an internal standard and related to the total neutral lipid fraction (33% of total lipids). Percentages of individual phospholipids were calculated from duplicate phosphorous determinations of phosphatides resolved by TLC and are not corrected for differences in molecular weight.

Fatty acid composition of individual lipid classes. The percentage distribution of fatty acids according to chain length and degree of unsaturation found in each lipid class isolated from the membranous material is given in Table II. The major fatty acids in all the lipid classes except phosphatidyl inositol (PI) and sphingomyelin were 16:0, 18:0, 18:1, 18:2, and 20:4. Cholesterol esters contained a lower percentage of polyunsaturated acids and a higher percentage of saturated acids than sterol esters from the whole cell (5). The level of 16:1 in the cholesterol esters of the membranous material was higher than that of the other lipid classes or the cholesterol esters of the whole cell. High levels of 16:1 in sterol esters of membranous material, first observed in the rat liver plasma membranes (30), appear to characterize the sterol esters of this cel

TABLE II

FATTY ACID COMPOSITION OF NEUTRAL LIPID AND PHOSPHOLIPID CLASSES ISOLATED FROM EHRLICH ASCITES CELL MEMBRANE FRACTION

Lipid class	Percentages of fatty acids[a,b]										
	14:0	16:0	16:1	18:0	18:1	18:2	20:0	20:2	20:3	20:4	22:6
Chol. E.[c]	2	16	6	10	36	13	1	2	1	6	1
GEDE	2	31	2	13	19	16	1	2	1	6	3
TG	2	23	2	20	23	19	1	2	1	3	1
FFA	1	20	2	22	21	20	T	2	1	5	1
DPG	2	16	1	10	27	31	T	4	1	6	
PE	T	12	1	30	18	20	T	1	1	9	4
PS	2	9	1	49	13	9	1	T	2	3	3
PI	T	11		88			T				
PC	1	28	2	21	17	21		1	1	5	2
SPH[d]	3	47	T	16	1	T	2				

[a] Percentages represent the mean of duplicate analyses on a pooled sample of two membrane preparations from Ehrlich ascites cells obtained from approximately 100 mice.

[b] The difference between the sum of the percentages of each class and 100% represents the sum of minor percentages of other acids not given in the table.

[c] Abbreviations are: Chol. E., cholesterol esters; GEDE, glyceryl ether diesters; TG, triglycerides; FFA, free fatty acids; DPG, diphosphatidyl glycerol; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; PC, phosphatidyl choline; SPH, sphingomyelin; and T, represents less than 0.5%.

[d] Sphingomyelin fatty acids also included 22:0 (7%), 24:0 (8%), 24:1 (11%), and 24:2 (3%).

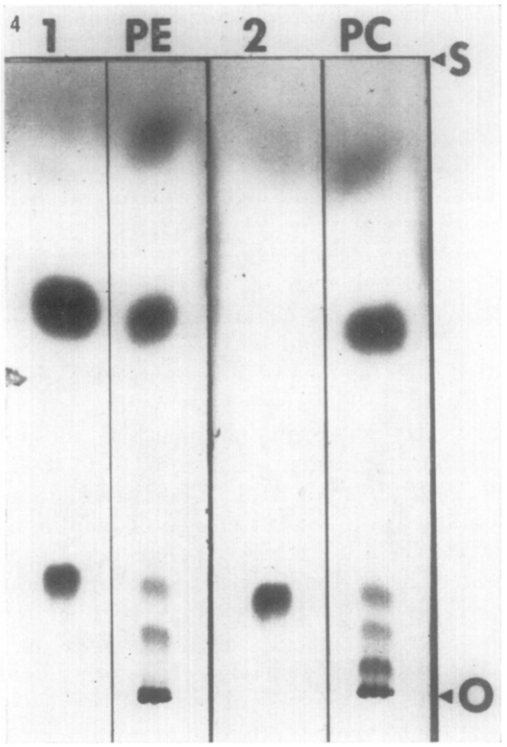

FIG. 4. Thin-layer chromatoplates of the hydrogenolysis products derived from PE and PC isolated from the membranous material. Lanes 1 and 2 contain alkyl glyceryl ether (lower spot) and long-chain alcohol standards. Alk-1-enyl glyceryl ethers migrate immediately above the alkyl glyceryl ether standard. The spots appearing between the alkyl glyceryl ethers and the origin are colored degradation products of fluorescent dyes. The origin and solvent front are indicated by O and S. Development was carried out in a diethyl ether–30% aqueous ammonium hydroxide (100: 0.5, v/v) solvent system.

structure. Triglycerides and glyceryl ether diesters contained approximately the same fatty acid distribution as the corresponding lipid classes obtained from whole cells (6) except for a decreased quantity of the 22:6 acid. Composition of the free fatty acids, a minor class of the whole cell lipids, was similar to PC and triglycerides.

Diphosphatidyl glycerol (DPG) contained the highest percentage of 18:2, a characteristic of DPG (7, 15, 30), but was approximately 10% lower than expected, on the basis of the mean of the 18:2 percentages at the 1- and 2-positions of DPG isolated from the whole cell (7). Percentages of the other acids were similar to the values obtained for DPG of the whole cell. The fatty acid percentages of PC and PE, the major phosphatides, were of the same order of magnitude as the corresponding mean percentages of the 1- and 2-positions of diacyl PC and PE obtained from whole cells (6). Similarity between PC and TG percentages agree with earlier data that indicated that 1,2-diglycerides of triglycerides and PC were similar in this neoplasm (6). Fatty acid composition of phosphatidyl serine (PS) isolated from the membranous material was very similar to the fatty acid percentages obtained for PS of whole Ehrlich ascites cells (7). Phosphatidyl inositol fatty acids were almost exclusively saturated, a sharp contrast to the composition of PI isolated from whole cells (7) where 20:4 acid represented two-thirds of the fatty acids esterified at the 2-position. This finding parallels data obtained for PI of rat liver where the 2-position of the plasma membrane PI contained a much higher percentage of saturated acids than PI of whole rat liver (15, 30). Sphingomyelin contained approximately 10% less 24:1 acid and a corresponding higher percentage of 16:0 acid than sphingomyelin obtained from whole cells (7). Sphingomyelin fatty acids of the membranous material contained the C_{24} dienoic acid, an acid that occurs at elevated concentrations in the sphingomyelins of neoplasms (7), but at a much lower level than that in the sphingomyelins of the whole cell.

Ether-linked lipids. The hydrogenolysis products of PC and PE are shown in Fig. 4 along with standards. Alk-1-enyl glyceryl ethers were not detected and only traces of alkyl glyceryl ethers were present in these two lipid classes isolated from the membranous material. Most of the material appearing on the sample lanes of Fig. 4 between the alkyl glyceryl ether and the origin is not due to carbon deposits of charred organic matter, but is due to yellow and red degradation products of the fluorescent dyes used to visualize the original phosphatides. Gas-liquid chromatographic analysis of isopropylidene derivatives prepared from the band opposite the alkyl glyceryl ether standard (Fig. 4) showed several peaks (20–30% of the total) in addition to the 16:0, 18:0, and 18:1 alkyl glyceryl ethers. On the basis of the assumption that all the eluting peaks were alkyl

glyceryl ethers, alkyl acyl PC and PE each represent only 1% of each class. The low level of the ether-linked lipids in the membranous material was unexpected in view of the fact that they have been shown to represent approximately 35 and 50% of PC and PE obtained from the whole Ehrlich ascites cells (6). Since it has been shown that the other phospholipid classes obtained from whole cells of this neoplasm contain only trace amounts of ether-linked lipids (7, 36), apparently the lipids of the membranous material do not contain significant quantities of ether-linked lipids. Lipids of rat liver plasma membranes have also been shown to be practically devoid of ether-linked lipids (30), but they have been shown to occur in membranous material of neural origin (37).

Further studies are required to determine their localization in these cells. The determination of the location of the ether-linked lipids in the cell may suggest a function of these lipids that now remains unknown.

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