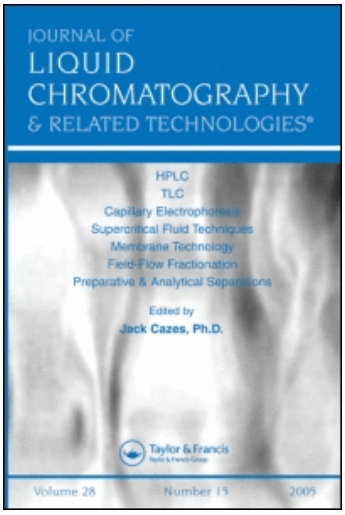

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Juan G. Alvarez[a]; Robert B. Zurier[b]; Bayard T. Storey[a]; John S. Bomalski[c]; Joseph C. Touchstone[a]
[a] Department of Obstetrics and Gynecology and of Physiology, University of Pennsylvania, Pittsburgh, Pennsylvania [b] Department of Medicine, Rheumatology Section, University of Pennsylvania, Pittsburgh, Pennsylvania, 19104 [c] Veterans Administration Medical Center and Medical, College of Pennsylvania, Philadelphia, Pennsylvania

To cite this Article Alvarez, Juan G. , Zurier, Robert B. , Storey, Bayard T. , Bomalski, John S. and Touchstone, Joseph C.(1988) 'Chromatographic Analysis of Phospholipids during Monocyte Maturation', Journal of Liquid Chromatography & Related Technologies, 11: 2, 395 — 415
To link to this Article: DOI: 10.1080/01483918809349948
URL: http://dx.doi.org/10.1080/01483918809349948

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(2), 395-415 (1988)

CHROMATOGRAPHIC ANALYSIS OF PHOSPHOLIPIDS DURING MONOCYTE MATURATION

Juan G. Alvarez[1], Robert B. Zurier[2], Bayard T. Storey[1], John S. Bomalski[3], and Joseph C. Touchstone[1]

[1]*Department of Obstetrics and Gynecology and of Physiology*
[2]*Department of Medicine, Rheumatology Section*
University of Pennsylvania
Pittsburgh, Pennsylvania 19104
[3]*Veterans Administration Medical Center*
and Medical College of Pennsylvania
Philadelphia, Pennsylvania 19104

ABSTRACT

Whether ether phospholipids are intrinsic to cell membranes or are acquired concomitantly with capacity for motion is unknown. Plasmalogen content was assessed in the human monocyte-macrophage-like cell line U937 that can be induced with dimethylsulfoxide (1.3%) to synchronously differentiate from poorly motile immature leukocytic cells to briskly motile mature cells. The phosphatidylethanolamine (PE) content of immature cells was 180 ± 10 $\mu g/10^8$ cells for U937 cells. Alkaline hydrolysis of PE resulted in 50% lyso-PE and 50% free fatty acid. Treatment of lyso-PE with acid and phospholipase A_2 demonstrated that PE is 1-(O-1'-aklenyl), 2-acyl PE. This was confirmed by infrared and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The phosphatidylcholine (PC) content of immature cells was 235 ± 5 $\mu g/10^8$ cells. In contrast to PE< 100% of PC was diacyl homologues. Following differentiation to mature motile cells, the plasmalogen content of PE

and PE significantly increased. The content of PC and PE in normal motile human peripheral blood monocytes was similar. The increase of plasmalogen in the PE component and its appearance in the PC component of mature motile U937 cells may indicate that plasmalogen correlates with the acquisition of motile function.

INTRODUCTION

Alkenyl ether phospholipids (plasmalogens) have been described in membranes of virtually all animal cells and most anaerobic bacteria (1-13). Tissue with high electrical activity such as rat and rabbit brain (6, 14-16), peripheral nerve (17) and myocardium (6, 18, 19) have a high alkenyl ether content, as do motile cells such as rabbit peritoneal polymorphonuclear leukocytes (20), rabbit alveolar macrophages (21), rabbit sperm (15), and the motile slime mold Dictyostelium discoideum (22). Therefore, a high plasmalogen content seems to be an important characteristic of electrically active tissues and mobile cells.

The ether phospholipid structure distinguishes plasmalogens from diacyl phospholipids (PL), and results in altered membrane architecture at the hydrophilic interface (23). Phospholipid phase behavior and cell membrane motion are dependent on phospholipid fatty acid interfacial regions (24), the nature of the fatty acyl chains (24), the polar head groups (24) and the sn-1 covalent linkage (25). Since these altered fluid phase characteristics of alkenyl ethers are unique to cells which exhibit excitation-contraction and electrical propagation and to motile cells, plasmalogens may be involved initiation of transmembrane ion movements, contractile apparatus function and receptor-ligand interactions.

An investigation of the plasmalogen content of the U937 human monocytic cell line was made to examine the role of plasmalogens in cell motility. When induced to differentiate terminally, these cells express enhanced chemotactic motility compared to their immature counterparts (reviewed in ref. 26). These cells were used to investigate plasmalogens in cell maturation that results in the capacity for motion. The existence and proportions of plasmalogens in the phospholipids of the human cells are reported here. An increase in the plasmalogen component of PE and the appearance of plasmalogen in PC in differentiated, motile, mature cells compared to their undifferentiated, poorly motile counterparts is described.

MATERIALS AND METHODS

Reagents

The synthetic standards 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanola-mine (DPPE), 1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol (DPPG), cardiolipin (CL), sphinogomyelin (SP), phosphatidylserine (PS), phosphatidylinositol (PI), lysophosphatidylcholine (LPC), cholesterol (Chol), trigylcerides (TG), fatty acids (FA), and cholesterol esters (CE) were obtained from Avanti Biochemicals (Birmingham, AL) or Nu-Chek-Prep (Elysian, MN). PE from bovine brain (PEB) was obtained from Supelco Inc. (Bellefonte, PA). Glycerylphosphoethanolamine (GPE), glycerylphosphocholine (GPC) and phospholipase A_2 from snake venom (Naja Naja) (E.C. 3.1.1.4) were purchased from Sigma Chemical Co. (St. Louis, MO). The Schiff reagent was obtained from Accra-Lab Inc. (Bridgeport, NJ).

Standard solutions were prepared at concentrations between 0.5 and 2 mg/ml in chloroform/methanol (1:1, v/v).

Solvents were EM Science chromatographic grade. Pre-coated LK5 (Silica) plates (250 μm thick) with preabsorbent zone of 500 μm thickness were obtained from Whatman Inc. (Clifton, NJ). Inorganic salts were from J.T. Baker (Phillipsburg, NJ) and of the highest purity available. Ficoll-Hypaque was obtained from Pharmacia (Piscataway, NJ), and phosphate buffered saline, RPMI and Dulbecco's modified Eagles' media, glutamine, fetal calf serum, trypan blue, penicillin and streptomycin were obtained from GIBCO (Grand Island, NY). Nonspecific esterase was obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of U937 Cells

Cells were obtained from Drs. Giorgio Trinchieri and Bruce Freundlich of the Wistar Institute and University of Pennsylvania. They were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS), minimal essential amino acids, glutamine (4 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Grand Island Biological Co., Grand Island, NY) at 37°C in a 5% CO_2/95% air incubator, and used when in the log phase of growth. Cells were induced to differentiate with 1.3% (v/v) dimethylsulfoxide (DMS) (27), and differentiation was monitored by nonspecific esterase staining, growth inhibition and antibody dependent cellular cytotoxicity as described by this laboratory (28, 29).

Human Monocyte Isolation

Peripheral blood monocytes were obtained from normal human volunteers using standard Ficoll-Hypaque centrifugation techniques followed by elution from plastic flasks coated with gelatin and serum (28). Cells were pooled, centrifuged, and resuspended in phosphate buffered saline (PBS) for immediate analysis. 95% were usually positive for nonspecific esterase. Cell viability as determined by trypan blue exclusion was greater than 95%.

Thin Layer Chromatography of Phospholipids

Whatman LK5 silica gel plates (20 x 20 cm; 250 μm thick with preadsorbent zone) were washed by continuous development overnight in chloroform/methanol (1:1, v/v). Those plates used for in situ chemical and enzymatic hydrolysis experiments were scored on a Schoeffel scoring device to give 1 cm lanes prior to the washing procedure.

Samples of cell suspensions were applied directly to the preadsorbent zone in 125 μl aliquots. Four plates were used in each determination. Synthetic or purified PL standards were cochromatographed for reference. The plates were dried under a stream of warm air for 10 min, then predeveloped 3 times in chloroform/methanol (1:1 v/v) to the interface of the preadsorbent zone. Between each predevelopment the layers were air dried to evaporate the solvent. This procedure extracts PL from the sample and deposits it on the starting point (31).

The mobile phase was chloroform/ethanol/triethylamine/water (30:34:30:8, v/v/v/v) (32). Development proceeded to 2 cm from the top of the plate (approximately 1.5 hr). The plates were then dried and zones containing the standards were cut apart and dried in an oven at 170°C for 2 min. One strip was sprayed with a 10% solution of $CuSO_4$ in H_3PO_4. Th other was sprayed with a 0.2% solution of ninhydrin in acetone. The $CuSO_4$-sprayed plates were dried for 5 min at room temperature, heated in an oven at 100°C, and finally placed in an oven at 170°C for 10 min (31). Plates sprayed with ninhydrin were placed directly in an oven at 110°C for 5 min.

A Kontes Fiber Optic Scanner (Model 800) using a 440 nm filter was used for scanning in the double beam mode using transmission. A Hewlett-Packard 3390A integrator provided integration. DPPE, DPPC, LPE, LPC, PEB, LPEB, GPE and palmitic acid were used to obtain standard curves. Amounts of PL between 0.5 -2.0μg gave linear standard curves, so procedures were adjusted so that the amounts applied to the plates were in this range. The unknowns were interpolated directly form the standard curves for quantitation.

Spectroscopic Analysis of Phosphatidylcholine from U937

Phosphatidylethanolamine and phosphatidylcholine from U937 cells (PE-U937 and PC-U937) were isolated directly from the plates by scraping off the spots located by CuSO4 and ninhydrin staining (15). PL release from the silica gel was facilitated by adding a few drops of water, followed by 3 ml of chloroform/methanol. The suspension was shaken in a Vortex mixer, then centrifuged at 600X g for 5 min to remove silica.

This was repeated. The chloroform/methanol extract was filtered through a Millipore 0.45 μm membrane to remove residual silica and evaporated under nitrogen until the solvent was completely removed. Recovery was 90%. Samples then were subjected to ^{1}H-NMR and infrared analysis.

Infrared spectrometry was performed by applying the lipid sample in chloroform as a film across the window area of a 100 mg KBr pellet and allowing the solvent to evaporate. The pellet was placed in a micro holder and scanned with a Perkin Elmer 421 infrared spectrometer. Interpretations of the spectra were based on the observations of Lammers et al. (33) and Meakings (34), and individual characteristics of functional groups were assigned as described (35-37).

^{1}H-NMR analysis was carried out in a Brucker WH-360 spectrometer. All samples wre dissolved in $CDCl_3$. Chemical shifts are quoted in ppm downfield form the internal tetramethylsilane (TMS) standard and are accurate to within 0.005 ppm. The spectrometer was operated at 360 MHz. ^{1}H-NMR interpretations are based on the observations of Lammers et al. (33) and Hauser et al. (38).

Plasmalogen Identification by Acid Hydrolysis

Phospholipids were subjected to mild acid hydrolysis to give the corresponding glycerol and aldehyde. This provides a rapid method for plasmalogen identification (15, 38). The reaction was carried out in situ by streaking the sample on the preadsorbent zone of scored K5 plates followed by 25 μl of a mixture of 2% trichloracetic acid and 8% hydrochloric acid (TCA/HCl) 1:1, v/v) and kept at ambient conditions for

10 min. Then 2 predevelopments and the final development of the chromatogram were carried out as described above. After development, the plates were dried and one strip was cut from each edge of the plate in such a way that 2 lanes would be on each strip. These strips were dried in an oven at 170°C for 2 min. One strip was sprayed with $CuSO_4$ and the other with ninhydrin reagent, as described. The solvent front area was sprayed with a solution of Schiff reagent (40) for aldehyde identification. Quantitation of the aldehyde was done only on the $CuSO_4$-sprayed plate. The products of hydrolysis then were scraped individually from the remaining unstained plate and subjected to either a second acid alkaline or enzymatic hydrolysis. The identical procedure was used for purified PE-brain as a control.

Alkaline Hydrolysis

Phospholipids were subjected to mild alkaline hydrolysis to which the acyl groups are selectively sensitive. Products are the corresponding glycerol and fatty acid derivatives. Alkyl ether and alkenyl ether groups are not affected by this treatment (15, 39). The reaction was carried out *in situ* by streaking 10-20 µl of the cell suspension on the preadsorbent zone of the layer. Then 25 µl aliquots of a mixture of methanol and 2.4 N sodium hydroxide (2:1), with final concentration 0.8 N, were applied over the areas of sample. Five ml of a methanol-water mixture (1:2) were placed in standard size tanks and equilibrated in an incubator at 37°C to saturate the tank with methanol-water vapor. Immediately after addition of the 25 µl of the NaOH/methanol mixture to the sample, the plates were placed in the presaturated tanks and incubated for 20 min. After incubation, plates were predeveloped in chloroform-methanol (1:1) followed by development.

Phospholipase A_2 Hydrolysis

Enzymatic hydrolysis were performed directly on the silica plate in exactly the same manner as for acid or alkaline hydrolysis. After separation of the Pl in the 8 lanes, 25 µl of phospholipase A_2 solution (5 mg/ml) in PBS with 2.5 mM $CaCl_2$ (40) was added to each lane and kept at room temperature for 10 min. The reaction mixture containing PLA_2 remained at pH of 7.4 ± 0.2, as tested in control lanes, throughout the reaction period. The plates were dried, treated to develop the chromatogram, and stained with $CuSO_4$ and ninhydrin.

Phospholipid Phosphorus

PL phosphorus was determined as described (41).

Statistics

Student's t-test for pairs was used.

RESULTS AND DISCUSSION

The phospholipid profile in the monocyte undifferentiated cell lines U937 are shown in Figure 1 and Table 1. PC accounted for 39% of the PL in U937 (PC-U937). PC-U937 appears to be the diacyl form, since treatment with the NaOH reagent, which is selective for acyl moieties, resulted in complete hydrolysis to GPC and FA (100%). Treatment of PC-U937 with the TCL/HCl reagent did not affect PC (Figure 2), indicating the absence of alkenyl ether moieties. These results indicate that PC-U937 contains only diacyl homologues. When subjected to alkaline

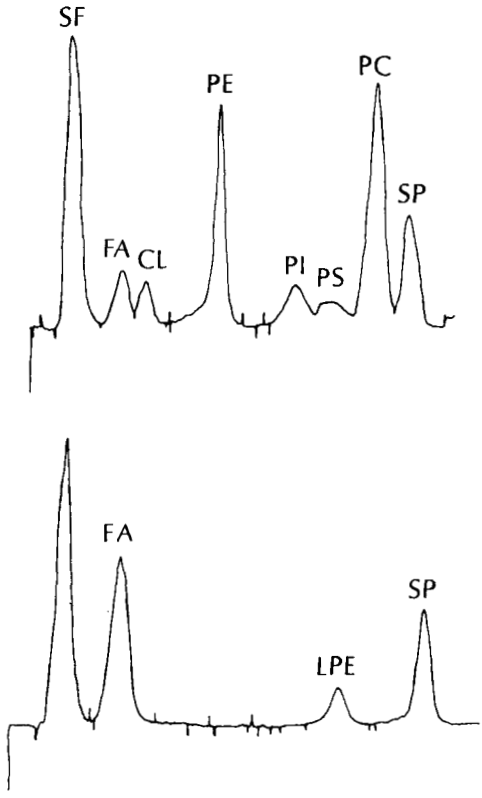

Figure 1: Densitometric scans from thin layer chromatography (TLC) plates on which phospholipids from immature monocytic U937 cells were separated, and stained with $CuSO_4$. The upper chromatogram shows the phospholipid profile obtained with immature monocytic U937 cells. The components identified by the labelled bands to the right of the front (SF) are: FA, fatty acid; CL, cardiolipin: PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidycholine; SP sphingomyelin. The lower chromatogram shows the phospholipid profile obtained after treatment with 0.8N methanolic NaOH.

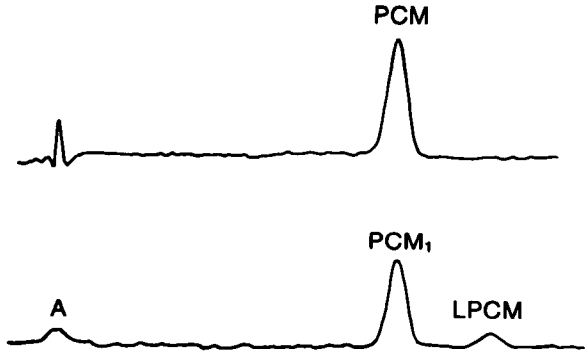

Figure 2: Densitometric scans of TLC plates with isolated immature monocytic U937 phosphatidylcholine (PCM) prior to and after treatment with the TCA/HCl reagent. The profile of untreated PCM is shown in the upper panel, after $CuSO_4$ staining; that of PCM treated with the TCA/HCl reagent and detected with the Schiff reagent is shown in the lower panel. PCM = phosphatidylcholine remaining after mild acid hydrolysis of PCM; LPCM = lysophosphatidylcholine. A = aldehyde.

TABLE 1. PHOSPHOLIPIDS IN MONOCYTES

U937 Cell Line

	μg PL/10^8 cells	μg P/10^8 cells
PC	135 ± 5	9.5 ± 1
PE	180 ± 1	7.3 ± 0.8
SP	85 ± 5	3.4 ± 0.5
PI	44 ± 3	1.8 ± 0.1
PS	19 ± 1.7	0.8 ± 0.08
CL	18 ± 3	0.7 ± 0.05

hydrolysis, PE-U937 yielded LPE (50%) and FA (50%), indicating that all PE-U937 molecules contained 1 acyl moiety. Further treatment of LPE with the TCL/HCl reagent resulted in GPE and free aldehyde (Figure 3). These results indicate the presence of acyl (50%) and alkenyl ether (50%) moieties in PE-U937, and the absence of alkyl ether moieties. Treatment of PE-U937 with PLA_2 resulted in complete hydrolysis to LPE (50%) and FA (50%) indicating the presence of a acyl moiety, presumably at the 2 position by definition. A qualification to this conclusion is that commercial PLA_2 may be contaminated with lysophospholipase (42). These results indicate that PE-U937 is 1-(O-1'-alkenyl), 2-acyl PE.

Further support for these assignments for P-U937 and PE-U937 was shown in the ^{1}H-NMR and infrared spectroscopy. ^{1}H-NMR data for PC-U937 and PE-U937 are summarized in Table 2. While the CH_2 protons adjacent to the ester group and the vinyl protons adjacent to the ether oxygen were present in PE-U937, only the former were found in PC-U937. The presence of both acyl and alkenyl ether moieties in PE-U937, but only acyl moieties in PC-U937 is indicated.

The infrared spectra of PC-U937 and PE-U937 are shown in Fig. 4. The spectrum of PC-U937 showed a strong C=O absorption band at 1720 cm^{-1}, moderately strong C-O stretching vibration at 1195 cm^{-1} and moderate P-O-C absorption at 1040 cm^{-1}. PE-U937 showed two differences when compared to PC-U937: the C=O absorption band was weak to moderate and the presence of the CH=CH-O absorption band at 815 cm^{-1} which was absent in PC-U937 (Table 3). This provides additional evidence for the characterization of PC-U937 as 1,2 diacyl PC and PE-U937 as 1-(O-1'-alkenyl), 2-acyl PE. DMSO differentiated cells were examined (26) to

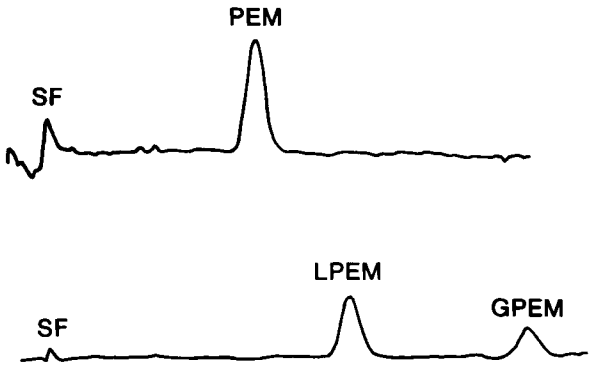

Figure 3: Densitometric scans of TLC plates with immature monocytic U937 phosphatidylethanolamine (PEN) prior to and after treatment with the TCA/HCl reagent, and detection with $CuSO_4$ staining; that of PEM treated with the TCA/HCl reagent and stained with ninhydrin is shown in the lower panel. LPEM = lysophosphatidylethanolamine; GPEM = glycerylphosphoethanolamine.

determine if cellular differentiation and capacity for motion correlated with changes in PL and plasmalogen concentration. The PE, PC and plasmalogen contents of undifferentiated (immature) and DMSO stimulated (mature, terminally differentiated) U937 cells are compared in Table 4. Although total PE and PC content was not altered when cells were differentiated with DMSO, the more mature cells exhibited changes in PE and PC plasmalogen. PE plasmalogen increased from 90 ± 5 to 142 ± 12 $\mu g/10^8$ cells; 10 ± 3 $\mu g/10^8$ cells of PC plasmalogen was found in mature cells. Increased production of plasmalogen or redistribution of this ether lipid is associated with differentiation of this particular cell

TABLE 2. PROTON NUCLEAR MAGNETIC RESONANCE SPECTRA OF PC-U937 AND PE-U937 WITH ASSIGNMENTS TO FUNCTIONAL GROUPS OF THE PHOSPHOLIPIDS

Compound[b]	CH_3	$(CH_2)n$	3-CH_2	β-CH_2	α-CH_2	2-CH	1-CH
I	0.88	1.28	N.D.	1.60	2.5	N.D.	N.D.
II	0.88	1.28	2.01	1.60	2.5	5.36	5.9

a: Positions of methylene and methine groups on acyl and alkenylether chains are defined as:

$$-O-C-\alpha CH_2-\beta CH_2-(CH_2)n-CH_3 \qquad -O-CH=CH-CH_2-(CH_2)n-CH_3$$

$$1 \quad 2 \quad 3$$

methyl group is terminal methyl of chain.

b: I. Phosphatidylcholine from U937 (PC-U937).

II. Phosphatidylethanolamine from U937 (PE-U937).

c: Not detectable.

TABLE 3. ABSORPTION BANDS AND THEIR ASSIGNMENTS IN THE INFRARED SPECTRA OF PC-U937 and PE-U937

Compound[a]	C=O	C-H	CH=CH	C-O	P-O-C	CH=CH-O
I	1720	1450	1625	1200	1030	N.D.[b]
II	1720	1450	1625	1200	1030	810

a: I. Phosphatidylcholine from U937 (PC-U937).

II. Phosphatidylethanolamine from U937 (PE-U937).

b: Not detectable.

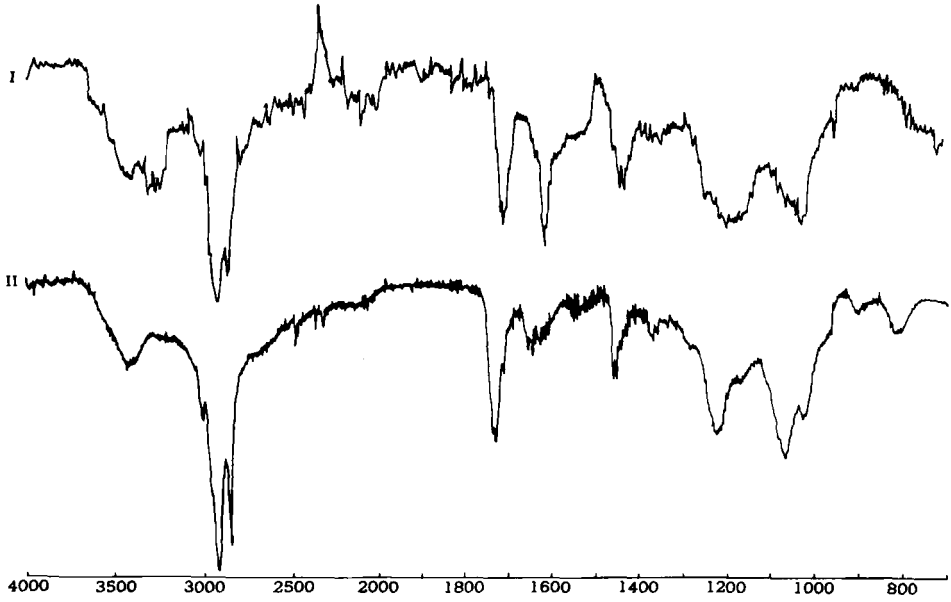

Figure 4: Comparison of the infrared spectra of PEM (I) and PEB (II). Wave numbers of maximum absorbance giving absorbance bands corresponding to the functional groups in both PE preparations are listed in Table 3.

I. Phosphatidylethanolamine from cultured immature monocytic U937 (PEM) cells.

II. Phosphatidylethanolamine from bovine brain (PEB).

TABLE 4. PLASMALOGEN CONTENT OF U937 CELLS*

	Total PL**	Plasmalogen	Total PL	Plasmalogen
Immature	180 ± 10	90 ± 5	235 ± 5	N.D.
Mature	180 ± 11	142 ± 12***	234 ± 4	10 ± 3***

* Expressed as content in $\mu g/10^8$ cells ± S.D., n = 5.

** PL = phospholipid

*** $p < 0.05$

N.D. = not detectable.

line to terminally differentiated mature monocyte-macrophage-like cells. The differentiated cells also acquire the capacity to move along a chemotactic gradient (26). The increase in plasmalogen in the PE component and the appearance of plasmalogen in the PC component of the mature motile cells provides evidence that plasmalogen incorporation in the PL of the membrane correlated with the acquisition of motile function.

These observations with the monocytic cell line were compared to the plasmalogen content of normal human monocytes. Normal fresh peripheral blood monocytes contain PE that is 100% plasmalogen and a PC component that is approximately 15% plasmalogen. In comparison rabbit alveolar macrophage PC contains 5.6 ± 1.2% plasmalogen, PE contains 61.2

± 4.2% (21). Rabbit peritoneal polymorphonuclear leukocytes elicited with Type II shellfish glycogen contain small amounts of alkenyl groups in PC and 63.4 ± 2.5% in PE (20). Thus, these three types of motile leukocytes from normal hosts all contain significant quantities of PE as plasmalogen and smaller amounts of PC as plasmalogen that correlated with enhanced formation of plasmalogen in U937 cells during differentiation. This cell line may be of value in investigating further the role of plasmalogens in cellular differentiation and motility.

ACKNOWLEDGEMENTS

Supported in part by NIH Grants HD-15842, HL-07027 and AM-28560, Grants from the Veteran's Administration and the Upjohn Company.

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