# Phospholipid Composition of the Granular Amebocyte from the Horseshoe Crab, *Limulus polyphemus*

**Jennifer C. MacPherson[a], James G. Pavlovich[b], and Robert S. Jacobs[a,*]**

[a]Marine Science Institute and [b]Department of Chemistry, University of California, Santa Barbara, California 93106

**ABSTRACT:** The phospholipid composition was determined for the amebocyte of the primitive arthropod *Limulus polyphemus*. The total fatty acid composition of the cells' lipids was analyzed by gas chromatography/mass spectrometry (GC/MS) of fatty acid methyl esters (FAME). The FAME analysis revealed high levels of 20-carbon polyunsaturated fatty acids (PUFA), especially arachidonic (20:4n-6) and eicosapentaenoic (20:5n-3) acids. Almost 20% of the total lipid profile was comprised of dimethyl acetals of 16- to 20-carbon chain lengths, indicative of plasmalogens in the phospholipid pool. Phospholipids, analyzed by high-pressure liquid chromatography, included phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SPH), and cardiolipin (CL). PE and PC levels predominated at 42.2 and 36.3%, respectively. Smaller amounts of PS (9.0%) and PI (6.2%) were present, as well as low levels of SPH (4.6%), CL (1.6%), and trace amounts of lysophosphatidylcholine. The major phospholipid species, PE, PC, PS and PI, were collected and their molecular species were examined by electrospray–ionization mass spectrometry. The molecular species within the phospholipid classes reflected the high levels of PUFA seen in the total lipid profile. PI was mainly composed of 18:0*a*/20:4. Over half of the PS consisted of 18:0*a*/18:1 and 18:0*a*/20:4. The major PE species were 20:1*p*/20:5, 20:1*p*/20:4, 18:0*p*/20:5, and 18:0*p*/20:4. PC had the largest distribution of molecular species, and its most abundant species were 16:0*e*/20:5, 16:0*e*/20:4, and 16:0*p*/20:4. The presence of 16:0*e*/20:4 is the first documentation of a specific precursor to platelet-activating factor in an invertebrate hemocyte. Note: at the *sn*-1 position: [*a* = 1=*O*-acyl, *e* = 1-*O*-alkylether, and *p* = 1-*O*-alk-1′-enyl (plasmalogen)].
*Lipids 33,* 931–940 (1998).

*To whom correspondence should be addressed.
E-mail: rsjacobs@chem.ucsb.edu

Abbreviations: AA, arachidonic acid; CL, cardiolipin; DMA, dimethyl acetal(s); EPA, eicosapentaenoic acid; ESI/MS, electrospray–ionization/mass spectrometry; FAME, fatty acid methyl ester(s); GC/MS, gas chromatography/mass spectrometry; HPLC, high-pressure liquid chromatography; $IP_3$, inositol-1,4,5-triphosphate; LPC, lysophosphatidylcholine; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid(s); SPH, sphingomyelin; phospholipid molecular species nomenclature, from Reference 1, *n:jk/s:t,* where *n* = number of carbons at the *sn*-1 position, *j* = number of double bonds in the *sn*-1 chain, *k* = the type of linkage at the *sn*-1 position [*a* = 1-*O*-acyl, *e* = 1-*O*-alkylether, and *p* = 1-*O*-alk-1′-enyl (plasmalogen)], *s* = number of carbons at the *sn*-2 position, and *t* = the number of double bonds in the *sn*-2 chain.

The horseshoe crab, *Limulus polyphemus*, serves as a unique model for studying the lipid chemistry of invertebrate blood cells. The majority of lipid analyses in arthropods have involved studies on whole animals, muscle tissue, or specific organs owing to constraints on organism size and the amounts of blood available (2,3). The presence of as many as six circulating blood cells in arthropods further complicates the isolation and analysis of an individual cell type (4). In horseshoe crabs, large quantities of blood are readily available and a single circulating blood-cell type, the granular amebocyte (5), is present. Moreover, *Limulus* is considered to be a living fossil with blood cells regarded as "primordial immunocytes" because they possess functions comparable to those found in vertebrate leukocytes, such as platelets, macrophages, and B- and T-lymphocytes (4). These vertebrate leukocytes are especially active during an immunological challenge brought on by injury or infection. The activation of these blood cells during an immune response is associated with the production of fatty acid metabolites, termed eicosanoids, which are primarily generated from unsaturated 20-carbon polyunsaturated fatty acids (PUFA) cleaved from the cell's phospholipid membrane, notably di-homo-γ-linoleic acid (20:3), arachidonic acid (AA) (20:4), and eicosapentaenoic acid (EPA) (20:5). The presence of these PUFA in specific phospholipid pools in the cell membrane also has been implicated in accelerating membrane fusion (6). The *Limulus* amebocyte produces eicosanoids (7), and its rapid degranulation in the presence of pathogens is well established (8,9). Therefore, a better understanding of the phospholipid composition, as well as the molecular species within each phospholipid class, will provide a framework for interpreting some of the functions of this primitive undifferentiated cell. In these studies, we will report the phospholipid composition of the *Limulus* granular amebocyte.

## MATERIALS AND METHODS

*Chemicals and materials*. The chloroform, methanol, water, hexane, isopropanol, and acetone used to extract, fractionate, and separate lipids were Fisher Scientific high-pressure liquid chromatography (HPLC)-grade (Pittsburgh, PA). The benzene was Fisher A.C.S.-certified grade and the ethanol was 200-proof dehydrated alcohol, U.S.P. punctilious (Quantum

Chemical Co., Anaheim, CA). Phospholipid, cardiolipin (CL), and sphingomyelin (SPH) standards were obtained from Sigma (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL).

*Horseshoe crabs.* The horseshoe crabs were purchased from either Marine Biological Laboratories (Woods Hole, MA) or Gulf Specimens (Panacea, FL) and maintained in tanks with fresh, flowing seawater and fed fresh local mussels, *Mytilus galloprovincialis,* collected by university divers from local piers. Blood samples were obtained within 1 wk of the animals' arrival. Animals with a prosoma, the larger anterior portion of the horseshoe crab which is followed by the smaller posterior opisthosoma, of 15–20-cm width were used.

*Cell isolation*. Prior to blood collection, the animals were cooled at 4°C for at 1 h. The animals were bled *via* cardiac puncture at the prosoma–opisthosomic joint using a sterile 16.5-gauge needle. Aliquots of blood (15 mL) were collected into sterile, silanized 50-mL Pyrex centrifuge tubes containing 10 mL of ice-cold saline (3.0% NaCl, buffered to pH 4.6 with citrate and EDTA) (5). The cells were briefly spun in a clinical centrifuge at room temperature (300 × *g*). The plasma/buffer supernatant was decanted, and the lightly pelleted cells were washed by resuspending in 10 mL of sterile 3% NaCl. Duplicate blood samples were obtained from each animal: one sample was used for fatty acid methyl ester (FAME) analysis and the other was extracted for phospholipid analysis. FAME were prepared directly from freshly isolated amebocytes to expedite analysis of the total lipid.

*FAME analysis*. The amebocyte's total cellular fatty acids were saponified and derivatized directly by a four-step process (10–12). The blood cells were isolated and washed, as detailed above, and then transferred into a 10-mL Pyrex test tube with a Teflon-lined lid and pelleted in a clinical centrifuge at room temperature (300 × *g*). The supernatant was decanted; the cells were lysed, and the lipids were saponified by adding 2 mL of 3.75 M NaOH/MeOH (50:50, vol/vol) and by heating at 100°C for 30 min. Samples were cooled, and the free fatty acids were methylated with the addition of 3 mL of 6 N HCl/MeOH (65:55, vol/vol) and with heating at 85°C for 10 min. The samples were cooled and 1 mL of hexane/$CH_2Cl_2$ (50:50, vol/vol) was added. The tubes were inverted end-over-end for 3–5 min. The aqueous layer was removed and 3 mL of 0.3 M NaOH was added. The tubes were inverted end-over-end for 10 min. The upper organic layer was transferred to an amber vial. The samples were placed under nitrogen for either immediate analysis by gas chromatography/mass spectrometry (GC/MS) or stored at −35°C until analyzed.

*GC/MS analysis*. FAME samples were analyzed on a Hewlett-Packard 5790 gas chromatograph (Palo Alto, CA) coupled to a VG 70SE double-focusing mass spectrometer using a DB-5MS column, 30 m × 0.25 mm i.d. (J&W Scientific, Folsom, CA). A 1-μL aliquot of the FAME sample was injected. The DB-5 column was held at 50°C for 1 min, ramped to 125°C at 18°/min, and then ramped to 255°C at 3°/min. The sample chromatograms were compared to an authentic standard of mixed FAME (PUFA-2) (Matreya Inc., Pleasant Gap, PA) run under the same conditions as the samples. The mass spectra of the dimethyl acetal (DMA) peaks were compared with known spectra National Institute of Standards and Technology (NIST) library.

*Lipid extraction and fractionation*. The cells for the phospholipid analyses, isolated as described above, were transferred to a 15-mL, silanized Pyrex test tube with a Teflon-lined cap. The cells were pelleted, the supernatant was decanted and then the cells were resuspended in 1 mL of sterile 3% NaCl. The cells were extracted using a modification of Kates (13). A 3.75-mL aliquot of methanol/chloroform (2:1, vol/vol) was added to the cell solution. The samples were vortexed and placed on a rotary shaker for 2 h. The solution was centrifuged in a clinical centrifuge for 5 min, the supernatant was transferred to a new tube, and the pellet was reextracted in 4.75 mL of methanol/chloroform/water (2:1:0.8, by vol). The sample was centrifuged, as before, and the supernatant was pooled with the first extract. A 2.5-mL aliquot each of chloroform and water was added to the pooled extract. The samples were vortexed, centrifuged, and the lower aqueous layer was removed. An equal volume of benzene was added and samples were taken to dryness under $N_2$. The samples were immediately resuspended in chloroform/MeOH (4:1, vol/vol) and stored under $N_2$ at −35°C until fractionated.

The samples were dried and resuspended in chloroform before being fractionated on small silica columns in Pasteur pipets using Baker-analyzed 60–200 mesh silica gel (Phillipsburg, NJ). The columns were equilibrated in chloroform. The sample was applied to the column followed by 4 column volumes (8 mL) of chloroform to elute the neutral lipids, 16 column volumes (32 mL) of acetone to elute the glycolipids, and finally 4 column volumes (8 mL) of MeOH to elute the phospholipids and SPH. The fractions were dried down under $N_2$ and stored at −35°C until analyzed.

*HPLC analysis of phospholipid classes*. The phospholipid extracts were analyzed using the method developed by Patton *et al*. (14). The lipid classes were separated using a Hitachi 6200A HPLC (San Jose, CA) equipped with two Rainin Silica Microsorb-MV columns (Woburn, MA) in tandem (4.6 i.d. × 150 mm, 5 μm, 100 Å). The columns were preceded by an Upchurch precolumn packed with 30–40 μm PerisorbA (Oak Harbor, WA). The phospholipid peak elution was monitored at 205 nm. The separation was isocratic starting with a flow rate of 0.5 mL/min for the first 60 min and then increased to 0.8 mL/min for the remainder of the run (*ca*. 180 min). The solvent phase was water/1 M phosphate buffer (pH 7.4)/isopropanol/-hexane/EtOH/acetic acid (55:1.2:485:376:100:0.6, by vol). The peaks for each lipid class were collected. CL samples were pooled while phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and SPH peaks were collected individually for electrospray–ionization/mass spectrometry (ESI/MS) analysis to determine their molecular species. Phosphate does not easily volatilize and therefore interferes with ESI/MS analysis. To avoid problems, samples were briefly taken to dryness under $N_2$ and reextracted to remove phosphate (13,15).

*ESI/MS analysis of molecular species within each phospholipid class*. The fatty acid composition of the major phospholipid classes (PE, PC, PS, and PI) was analyzed using a modification of Kim *et al*. (16). The phospholipid classes were separated on a Phenomenex C18 minicolumn (2.0 i.d. × 150 mm, 5 µm, 80Å) preceded by a 30-mm guard column of the same material using a Michrom BioResources UMA HPLC System (Auburn, CA) at a flow rate of 400 µL/m. The samples were separated using a starting solvent phase of 0.5% ammonium hydroxide in water/MeOH/hexane (12:88:0) for 3 min followed by a linear gradient to water/MeOH/hexane (0:18:12) over a 30-min period. The column was maintained at 20–25°C.

The solvent flow was split postcolumn (1:3, vol/vol), and 100 µL of the HPLC effluent was directed into the inlet of an ESI probe of a Fisons VG Platform II single quadrupole mass spectrometer (Micromass Ltd., Altrincham, United Kingdom). The ESI/MS was tuned daily by direct infusion of dipalmitoyl PC (positive-ion mode) or PI (negative-ion mode) (2 µM). The skimmer cone voltage was set during tuning for moderate fragmentation (−60 to −70 V in negative-ion and 40–50 V in positive-ion mode). The mass collection range was set between 100 and 1000 amu for all samples. Sample ions were generated using $N_2$ nebulization-assisted electrospray with a source temperature maintained at 80°C. All spectra were collected in total scan mode and subjected to background subtraction.

PI and PS samples were analyzed in negative-ion mode. PE and PC peaks were analyzed in both positive- and negative-ion modes. The negative-ion analyses of PE and PC were used to determine the fatty acids associated with the major molecular ions generated in the positive-ion mode. A phospholipid molecular ion can represent one, sometimes two, but rarely all three of the phospholipid subclasses (diacyl, alkylacyl, and alkenacyl). The molecular species of the major peaks were identified through examination of their mass spectra. The remaining molecular species listed were calculated based on the molecular ion and any available fragmentation data from the negative-ion spectra (1). The molecular ion usually represents the entire phospholipid, either $[M + H]^+$ or $[M - H]^-$, depending on the analysis mode, the exception being PC in negative-ion mode that displays a pseudomolecular ion of [M − 15], corresponding to a loss of a methyl group from the choline headgroup (17). Since the phospholipids were separated by class prior to ESI/MS, the headgroup is known and any free fatty acids present in the mass spectra are generated from ester linkages at the *sn*-1 or *sn*-2 position. The FAME/DMA profile in the GC/MS analyses was also used to predict the likely diacyl and alkenylacyl species present in each sample. In the more complicated phospholipid classes, PE and PC, published tables of the three subclasses (diacyl, alkylacyl, and alkenylacyl) were used to help determine the molecular species of the minor peaks (18). In these tables, the molecular ion is calculated based on *sn*-1 and *sn*-2 linkages, as well as the total number of carbons and double bonds present in these positions. Since the double bond in the vinyl linkage of alkylacyl lipids is considered part of the linkage, it is not counted as a double bond in these tables (15,18).

*Analysis of smaller phospholipid peaks.* The smaller peaks present in the HPLC phospholipid separation (CL and SPH) were screened to identify major species present. The samples were separated by HPLC in a manner similar to the major phospholipid classes. The mass range was set between 100 and 1000 amu, except for CL samples where the range was extended to 2000 amu. These samples were screened in both positive- and negative-ion modes.

## RESULTS

*GC/MS analysis of FAME*. The chromatogram of the FAME revealed that almost half (46%) of the amebocyte fatty acid content consisted of two 20-carbon PUFA, AA, and EPA (Table 1). The next most abundant species were the saturated and monounsaturated 18-carbon fatty acids at 12.9 and 11.6%, respectively. DMA made up nearly 20% of the total lipid. DMA are formed by the acid methanolysis of phospholipids containing vinyl groups in ether linkages. Therefore, the DMA here indicate that a considerable portion of the phospholipids contains ether lipids with alkenyl groups at the *sn*-1 position of the phospholipids. The DMA profile in the amebocyte indicates that eicosene (20:1) was the most com-

**TABLE 1**
**Fatty Acid Composition of the *Limulus* Amebocyte[a]**

| Fatty acid | Percentage area |
|---|---|
| 14:0 | 0.1 ± 0.1 |
| 15:0 | trace |
| 16:0 | 2.2 ± 0.2 |
| 16:1n-7 | 0.2 ± 0.1 |
| 16:0 DMA | 1.4 ± 0.2 |
| *i/ai* 17:1 or 17:1 | 0.2 ± 0.0 |
| *i/ai* 17:0 | 0.2 ± 0.0 |
| 17:0 | 0.8 ± 0.1 |
| 17:0 DMA | 0.4 ± 0.1 |
| 18:0 | 12.9 ± 0.4 |
| 18:1n-9 | 8.4 ± 0.5 |
| 18:1n-7 | 3.2 ± 0.2 |
| 18:2n-6 | 1.1 ± 0.2 |
| 18:0 DMA | 5.9 ± 0.5 |
| 18:1 DMA | 0.7 ± 0.1 |
| 19:1, *i/ai* 19:0[b] | 3.9 ± 0.4 |
| 19:0 | 0.2 ± 0.1 |
| 20:0 | trace |
| 20:1n-9 | 0.8 ± 0.3 |
| 20:4n-6 | 25.4 ± 1.0 |
| 20:5n-3 | 20.5 ± 0.8 |
| 20:0 DMA | 0.5 ± 0.1 |
| 20:1 DMA | 9.5 ± 1.3 |
| 22:0 | trace |
| 22:4n-6 | 0.7 ± 0.1 |
| 22:5n-3 | 0.7 ± 0.1 |

[a]Values are the mean ± SEM, $n = 5$. Samples were derived from five separate individuals. Trace levels of fatty acid were considered to be less than 0.1%.
[b]This peak was a mixture of three molecular ions: 294, 310, and 312 amu.
DMA = dimethyl acetal; *i/ai* indicates iso- and anteiso-fatty acids.

**TABLE 2**
**Phospholipid Composition of the *Limulus* Amebocytes[a]**

| Phospholipid class | Percentage area |
|---|---|
| Phosphatidylethanolamine | 42.2 ± 1.0 |
| Phosphatidylinositol | 6.2 ± 0.5 |
| Phosphatidylserine | 9.0 ± 0.7 |
| Cardiolipin | 1.6 ± 0.5 |
| Phosphatidylcholine | 36.3 ± 0.7 |
| Sphingomyelin[b] | 4.6 ± 0.5 |

[a]The percentage area is the mean ± SEM of amebocyte preparations from seven animals.
[b]One of the samples analyzed by electrospray–ionization/mass spectrometry (ESI/MS) contained lysophosphatidylcholine.

mon alkyl chain in ether linkage (9.5%), with lesser amounts of octadecane (18:0) and hexadecane (16:0), 5.9 and 1.4%, respectively.

*HPLC analyses of the phospholipid classes.* A clean separation existed between the major phospholipid classes. Analysis of the phospholipid fraction revealed a greater proportion of PE (42.2%) than PC (36.2%) (Table 2), followed by lower levels of PS and PI at 9.0 and 6.2%, respectively. SPH (4.6%) and CL (1.6%) were also present in the amebocyte membranes. Lysophosphatidylcholine (LPC) was detected at trace levels in one of the SPH samples.

*ESI/MS analyses of the molecular species within the major phospholipid classes*. The levels of EPA and AA in the FAME analyses were reflected by the predominance of phospholipid species containing these fatty acids. To distinguish between alkyl and alkenyl linkages at the *sn*-1 position using ESI/MS without prior separation of these subclasses (13) is not possible, as previously stated, but the phospholipid molecular species can still be calculated based on the molecular ion, the headgroup, and any fatty acids that have fragmented from the phospholipid molecule, as seen in the negative-ion spectra. The diacyl and plasmalogen species agreed well with the species of FAME and DMA present in the total lipid (Table 1).

Examination of the negative-ion mass spectra for PI revealed only the molecular ion $[M - H]^-$ (Fig. 1A). To examine the fatty acid profile in the PI class, the sample also was analyzed at higher voltages (–80 to –100 V, data not shown). These spectra indicated 18-carbon *m/z* 281 (18:1) and 283

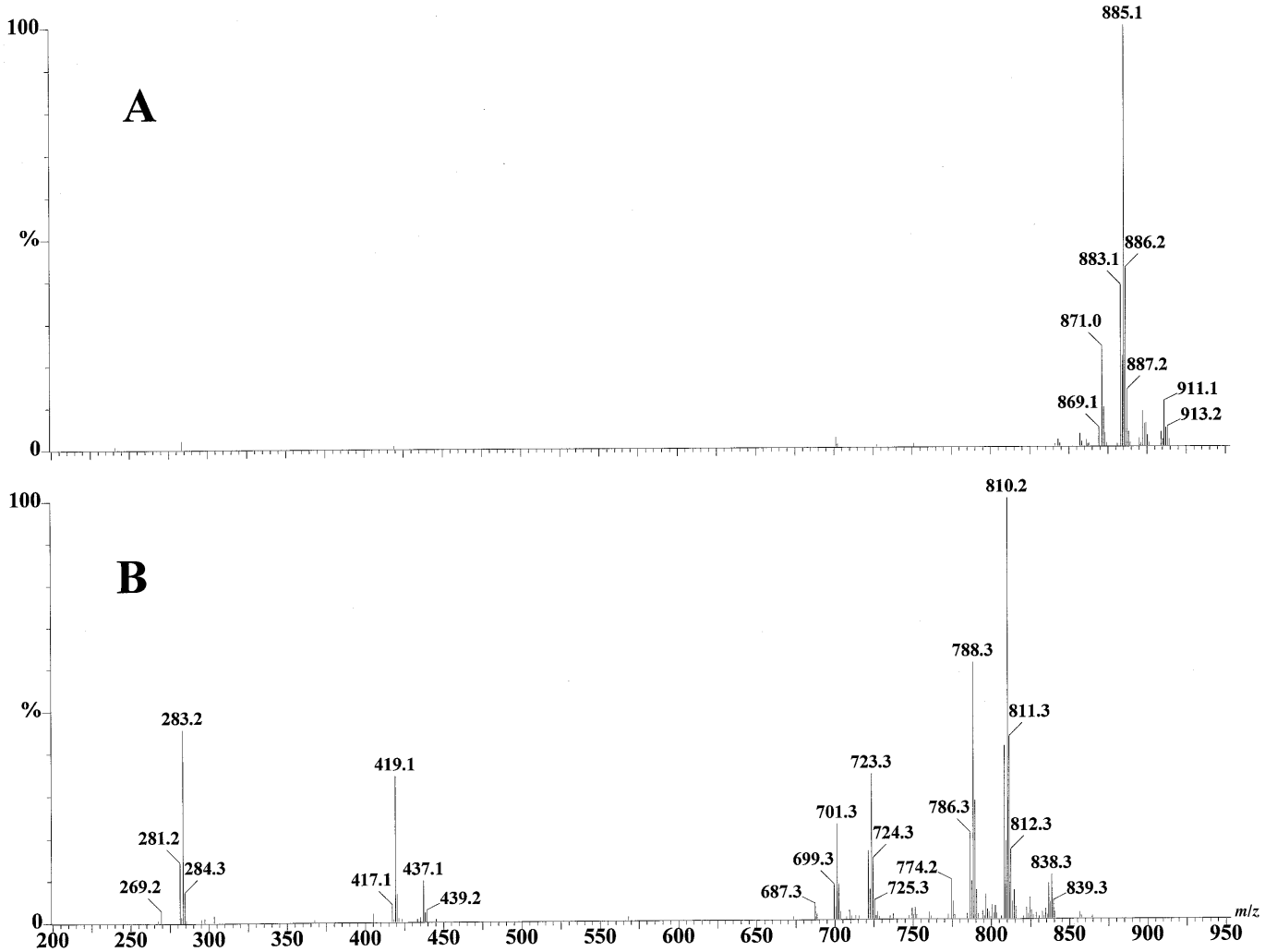


**FIG. 1.** Electrospray–ionization/mass spectrometry negative-ion mass spectra of the molecular ions and fragments of (A) phosphatidylinositols and (B) phosphatidylserines in the *Limulus* amebocyte.

(18:0), 20-carbon *m/z* 301 (20:5), 303 (20:4), 305 (20:3), 307 (20:2) and 309 (20:1), as well as *m/z* 313 (22:6), 315 (22:5), and 317 (22:4) were present. Greater than half of the PI (52.3%) consisted of a single species, 18:0*a*/20:4 (Table 3), where *a* = 1-*O*-acyl. Almost another quarter of the PI (21.4%) was composed of a mixture of the two species 18:0*a*/20:5 and 18:0*e*/20:4, where *e* = 1-*O*-alkylether. The PS negative-ion mass spectra displayed numerous fragments (Fig. 1B). The molecular ion $[M - H]^-$ was present, as well as ions corresponding to the loss of serine $[M - 88]^-$, and the loss of both serine and fatty acids (*m/z* 417–439) and free fatty acids (*m/z* 281, 283). There were two predominant species of PS, 18:0*a*/20:4 (*m/z* 810) and 18:0*a*/18:1 (*m/z* 788), at 33.8 and 22.7%, respectively (Table 3). The fatty acid composition of PS and PI species indicates that almost 90% of these lipids are diacyl species.

Analysis of the positive-ion mass spectra of the PE revealed the molecular ion $[M + H]^+$ and the loss of ethanolamine from the major species $[M - 142]^+$ (Fig. 2A). The negative-ion mass spectra (Fig. 3A) gave a molecular ion at $[M - H]^-$, as well as free fatty acids at *m/z* 281, 301, and 303. In contrast to the prevalence of diacyl species in the PI and PS fractions of the amebocyte, the PE molecular species consisted of plasmalogen (62%), diacyl (27%), and alkylacyl (11%) phospholipids (Table 4). The chains in the *sn*-1 position of ether lipids are in ether or vinyl ether linkage and are not readily cleaved from the phospholipid backbone during ESI/MS analysis. The general lack of saturated and monounsaturated 16- to 20-carbon free fatty acids in the negative-ion spectra further supports the predominance of ether lipids in this class. The major species were 20:1*p*/20:5 (27.3%) and 20:1*p*/20:4 (13.6%), where *p* = 1-*O*-alk-1′-enyl (plasmalogen). A significant amount of 18:0*p*/20:5 (13.7%) and 18:0*p*/20:4 (4.5%) also existed.

The positive-ion mass spectra for the PC yielded only molecular ions $[M + H]^+$ and the choline headgroup (*m/z* 184) (Fig. 2B). The negative-ion spectra for the PC were quite complicated. The pseudomolecular ion occurred at $[M - 15]$, which is indicative of demethylation of the choline headgroup (17) (Fig. 3B). The other peaks present included the losses of the quaternary nitrogen from the molecular ion (*m/z* 660–720), the loss of quaternary nitrogen and fatty acids (*m/z*

**TABLE 3**
**Molecular Species Composition of the Amebocyte Phosphatidylserines (PS) and Phosphatidylinositols (PI) in Negative-Ion ESI/MS[a,b]**

| | Molecular species | *m/z* | Composition (%) |
|---|---|---|---|
| PS | 18:1*a*/18:1 | 786.3 | 4.7 ± 0.6 |
| | 18:0*a*/18:1 | 788.3 | 23.0 ± 1.4 |
| | 18:0*a*/18:0 | 790.3 | 2.7 ± 0.2 |
| | 18:0*a*/20:5 | 808.3 | 3.3 ± 1.2 |
| | 18:0*a*/20:4 | 810.3 | 34.1 ± 1.0 |
| | 18:0*a*/20:3 | 812.3 | 5.9 ± 0.1 |
| | 18:0*a*/20:2 | 814.3 | 4.7 ± 0.7 |
| | 18:0*a*/22:5 | 836.3 | 4.4 ± 0.3 |
| | 18:0*a*/22:4 | 838.3 | 6.9 ± 0.8 |
| | 18:0*e*/18:1,18:0*p*/18:0 | 774.3 | 2.8 ± 0.1 |
| | 18:0*e*/20:4 | 796.3 | 1.0 ± 0.2 |
| | 18:0*e*/20:3 | 798.3 | 0.7 ± 0.2 |
| | 18:0*p*/20:1 | 800.3 | 1.3 ± 0.2 |
| | 18:0*e*/20:1 | 802.3 | 1.5 ± 0.5 |
| | 18:0*p*/22:4 | 822.3 | 0.8 ± 0.1 |
| | 18:0*e*/22:4 | 824.3 | 2.2 ± 0.3 |
| PI | 16:0*a*/20:4 | 857.1 | 1.1 ± 0.4 |
| | 18:0*a*/18:2 | 861.1 | 0.6 ± 0.1 |
| | 18:0*a*/20:5 | 883.1 | 14.7 ± 1.3 |
| | 18:0*a*/20:4 | 885.1 | 52.3 ± 1.8 |
| | 18:0*a*/20:3 | 887.1 | 7.3 ± 0.4 |
| | 18:0*a*/22:6, 18:1*a*/22:5 | 909.1 | 1.2 ± 0.1 |
| | 18:0*a*/22:5 | 911.1 | 4.5 ± 0.3 |
| | 18:0*a*/22:4 | 913.1 | 2.0 ± 0.1 |
| | 16:0*e*/20:4 | 843.1 | 0.8 ± 0.1 |
| | 18:1*p*/20:4, 18:0*e*/20:5 | 869.1 | 1.4 ± 0.2 |
| | 18:0*e*/20:4 | 871.1 | 6.7 ± 0.6 |
| | 18:0*e*/20:3 | 873.1 | 1.0 ± 0.1 |
| | 18:0*p*/22:5 | 895.1 | 0.7 ± 0.0 |
| | 18:0*e*/22:5 | 897.1 | 3.3 ± 0.3 |
| | 18:0*e*/22:4 | 899.1 | 2.4 ± 0.3 |

[a]Phospholipid nomenclature from Reference 1. The letter designates the linkage at the *sn*-1 position in the phospholipid, where *a* = 1-*O*-acyl, *e* = 1-*O*-alkylether, and *p* = 1-alk-1′-enyl (plasmalogen).
[b]The percentage composition was based on the average relative intensities of each molecular ion from the analyses of four separate individuals, mean ± SEM. See Table 2 for abbeviations.

**TABLE 4**
**Molecular Species Composition of the Amebocyte Phosphatidylethanolamines in Positive-Ion ESI/MS[a,b]**

| Molecular species | *m/z* | Composition (%) |
|---|---|---|
| 16:0*a*/20:4 | 736.3 | 1.6 ± 0.4 |
| 16:0*a*/20:5 | 738.3 | 0.5 ± 0.1 |
| 18:1*a*/18:1 | 744.3 | 0.5 ± 0.2 |
| 18:0*a*/18:1 | 746.3 | 0.6 ± 0.1 |
| 18:0*a*/18:0 | 748.3 | 3.4 ± 0.5 |
| 18:1*a*/20:5 | 764.3 | 2.1 ± 0.2 |
| 18:0*a*/20:5 | 766.3 | 3.6 ± 0.6 |
| 18:0*a*/20:4 | 768.3 | 4.3 ± 0.6 |
| 18:0*a*/20:3 | 770.3 | 1.1 ± 0.1 |
| 18:1*a*/22:5 | 792.3 | 0.7 ± 0.1 |
| 18:1*a*/22:4, 18:0*a*/22:5 | 794.3 | 1.0 ± 0.1 |
| 18:0*a*/22:4 | 796.3 | 0.8 ± 0.1 |
| 20:0*a*/20:3 | 798.3 | 2.6 ± 0.4 |
| 20:1*a*/20:1 | 800.3 | 1.3 ± 0.2 |
| 20:0*a*/20:1 | 804.3 | 2.6 ± 0.2 |
| 16:0*p*/20:5 | 722.3 | 2.0 ± 0.5 |
| 16:0*p*/20:4 | 724.3 | 0.7 ± 0.1 |
| 18:0*p*/20:5 | 750.3 | 13.7 ± 0.9 |
| 18:0*p*/20:4 | 752.3 | 4.5 ± 0.3 |
| 18:0*e*/20:4 | 754.3 | 1.9 ± 0.2 |
| 18:0*e*/20:3 | 756.3 | 4.0 ± 0.4 |
| 18:0*e*/20:0 | 762.3 | 1.1 ± 0.2 |
| 20:1*p*/20:5 | 776.3 | 27.3 ± 2.8 |
| 20:1*p*/20:4 | 778.3 | 13.6 ± 0.9 |
| 20:0*e*/20:5 | 780.3 | 2.9 ± 0.2 |
| 20:0*e*/20:4 | 782.3 | 0.8 ± 0.5 |
| 20:1*e*/20:0 | 788.3 | 0.6 ± 0.1 |

[a]Phospholipid nomenclature from Reference 1. The letter designates the linkage at the *sn*-1 position in the phospholipid. See Tables 2 and 3 for abbreviations.
[b]The percentage composition was based on the average relative intensities of each molecular ion from the analyses of five separate individuals, mean ± SEM.

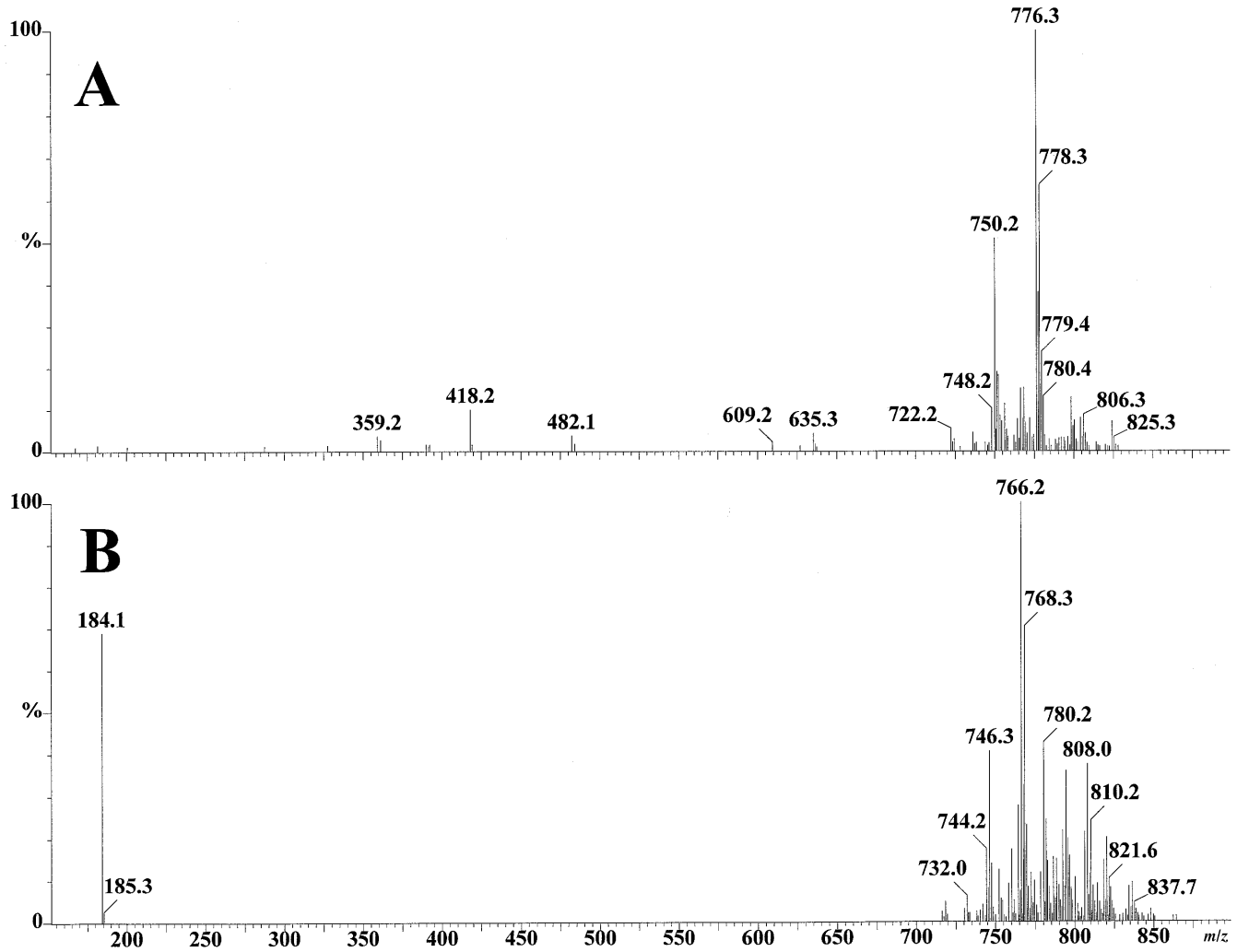


**FIG. 2.** Electrospray–ionization/mass spectrometry positive-ion mass spectra of the molecular ions and fragments of (A) phosphatidylethanolamines and (B) phosphatidylcholines in the *Limulus* amebocyte.

370–420), as well as the presence of large levels of free fatty acids (*m/z* 255–317). Whereas plasmalogen levels dominated in PE, the levels of the three subclasses in PC were almost evenly distributed. The composition of the PC species consisted of diacyl (39%), alkylacyl (35%), and plasmalogen (26%) phospholipids (Table 5). In comparison to the PE fraction, the presence of detectable 16:0 (*m/z* 255) and large 18:1 (*m/z* 281) in the negative-ion spectra mirrors the higher levels of diacyl phospholipid in the PC fraction. A broad distribution of molecular species was present in PC, but the three major species were a mixture of 16:0*e*/20:5 and 16:0*p*/20:4 (14.4%) and 16:0*e*/20:4 (8.9%).

*Screening of CL, SPH, and LPC species.* Small peaks in the phospholipid samples (<5%) were screened to identify major species present. The CL fraction was tested in both positive- and negative-ion modes (data not shown). In negative-ion mode, the mass spectra revealed mainly free fatty acids (20:5 > 20:4 ≫ 18:1 > 18:0 = 18:2 > 22:4 > 22:5). The positive-ion spectra showed a cluster of molecular ions in the range of *m/z* 1520–1660. The masses are most likely dipotassiated CL species resulting from the use of potassium phosphate in the HPLC solvent and potassium chloride in the wash buffer used to remove phosphate. The major masses present were *m/z* 1531, 1553, and 1579, indicative of CL with fatty acid mixtures of 18:1, 18:2, 20:4 and 20:5, which correlates with the fatty acid profile seen in the negative-ion mass spectra.

In the positive-ion mode scan of the SPH samples, an extracted ion profile of the choline headgroup, *m/z* 184, demonstrated the presence of six peaks (data not shown). The mass spectra of the five major peaks revealed apparent masses of *m/z* 675, 689, 703, 717 and 731, while the sixth minor peak had an apparent mass of *m/z* 661. Each of these apparent masses had an additional sodiated mass at $[M + 23]^+$. The incremental difference of 14 mass units between each major peak indicates a group of SPH with saturated carbon chains ranging from 13 (*m/z* 661) to 18 (*m/z* 731) carbons. In one of the four SPH samples tested, two minor LPC peaks were detected at *m/z* 496 (16:0 LPC) and *m/z* 524 (18:0 LPC). These LPC peaks displayed the characteristic cleavage of the

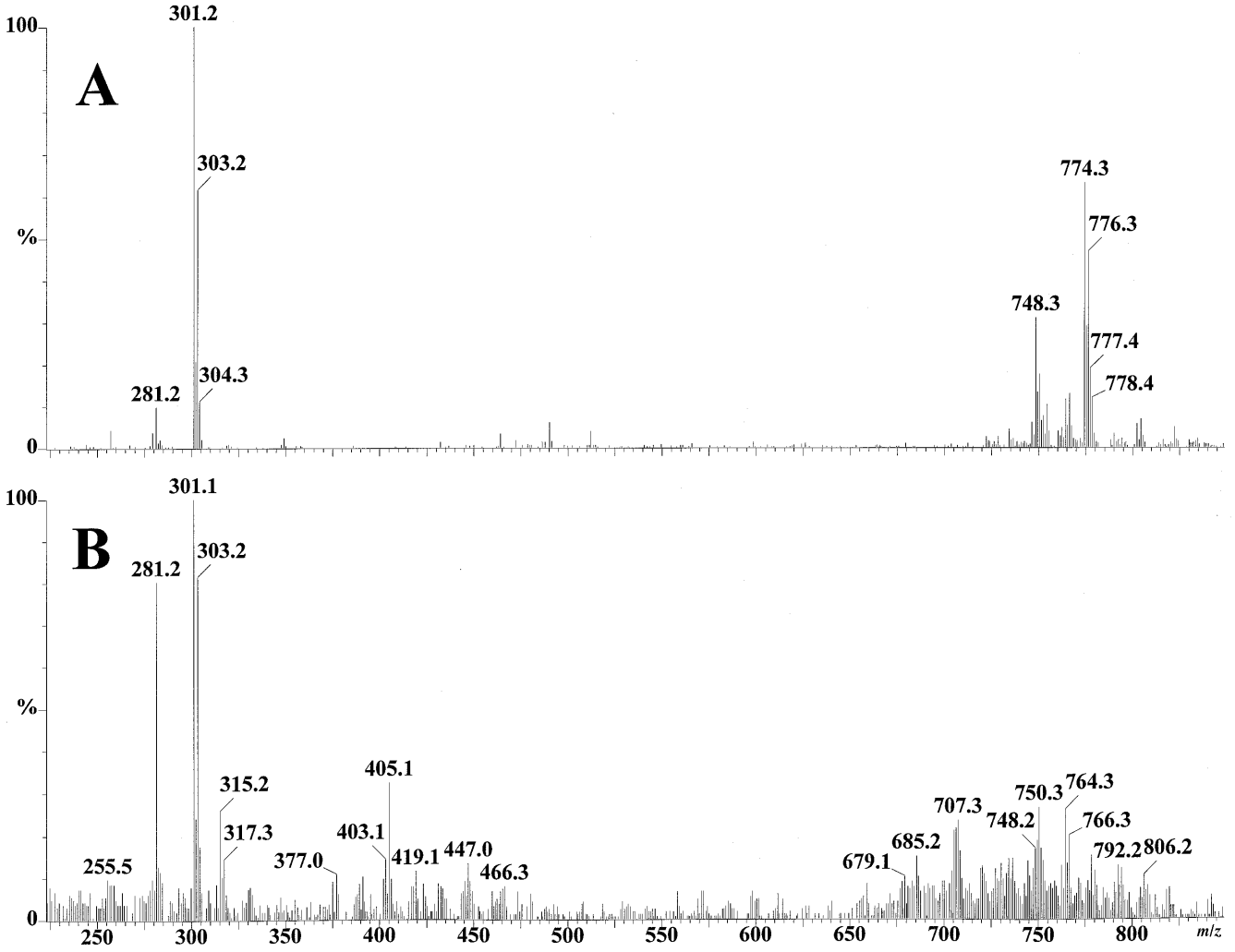


**FIG. 3.** Electrospray–ionization/mass spectrometry negative-ion mass spectra of the molecular ions and fragments of (A) phosphatidylethanolamines and (B) phosphatidylcholines in the *Limulus* amebocyte.

choline headgroup at *m/z* 184, the molecular ion $[M + H]^+$, the loss of water $[M - 18]^+$ peak, the addition of sodium $[M + 23]^+$, and the addition of potassium $[M + 38]^+$.

## DISCUSSION

*Analysis of FAME.* At present, very few reports are available on the lipid composition of arthropod blood cells to compare with that of the *Limulus* amebocyte. One report is available for the mixed crab blood cells of *Carcinus meanas* of 20% EPA and 10% AA levels (19). *Limulus* amebocytes demonstrate significant levels of ether lipids. Phospholipids with alkenylether bonds (plasmalogens), and to a lesser extent those with alkylether bonds, are also common components in vertebrate blood cells and are most often found in the PC and PE classes (20,21). Though the levels of ether lipids are modest in mammals, in lower animals they can reach appreciable levels, especially the alkylacyl phospholipids. Horseshoe crabs are arthropods, though more closely related to arachnids than crustaceans. In Sugiara *et al.* (2), the tissues of two arachnids, a spider and a water scorpion, were analyzed, and they contained between 6.7 and 17.9% alkylacyl PC and between 13.7 and 22.7% alkylacyl PE, respectively. A marine prawn contained 7% alkylacyl PC and 7% alkylacyl PE. Alkylacylphospholipids can represent up to 20% of the lipid in marine organisms, and in extreme cases as much as 50–80% (2,3,22). The most commonly reported groups in the *sn*-1 position of ether lipids are 16:0, 16:1, 18:0 and 18:1 (23); however, the DMA profile in the amebocyte indicates that eicosene (20:1) is the most abundant group in ether linkage (9.5%), with lesser amounts of 18:0 (5.9%) and 16:0 (1.4%). The role of eicosene ether lipids is not known, but they have been detected in small amounts at the *sn*-l position in the ether lipids of human erythrocytes, adrenal glands, and placentas (23). We also compared the amebocyte to vertebrate white blood cells with similar functions, i.e., platelets and macrophages.

*Analysis of the phospholipid class composition.* In comparison to mammalian leukocytes, the PE levels of the amebocyte (42.2%) were about 60% greater than those seen in

**TABLE 5**
**Molecular Species Composition of the Amebocyte Phosphatidylcholines in Positive-Ion ESI/MS[a,b]**

| Molecular species | *m/z* | Composition (%) |
|---|---|---|
| 16:0*a*/16:1 | 732.3 | 1.2 ± 0.2 |
| 14:0*a*/20:5 | 752.3 | 2.8 ± 0.5 |
| 14:0*a*/20:4 | 754.3 | 1.3 ± 0.2 |
| 16:0*a*/18:2 | 758.3 | 1.1 ± 0.0 |
| 16:0*a*/18:1 | 760.3 | 2.0 ± 0.1 |
| 16:0*a*/18:0 | 762.3 | 0.8 ± 0.1 |
| 16:1*a*/20:5 | 778.3 | 1.5 ± 0.1 |
| 16:0*a*/20:5 | 780.3 | 4.8 ± 0.2 |
| 16:0*a*/20:4 | 782.3 | 2.8 ± 0.4 |
| 18:1*a*/18:2 | 784.3 | 0.5 ± 0.1 |
| 18:1*a*/18:1 | 786.3 | 2.1 ± 0.1 |
| 18:0*a*/18:1 | 788.3 | 2.0 ± 0.2 |
| 18:0*a*/18:0 | 790.3 | 0.9 ± 0.2 |
| 18:1*a*/20:5 | 806.3 | 2.5 ± 0.1 |
| 18:1*a*/20:4, 18:0*a*/20:5 | 808.3 | 4.5 ± 0.2 |
| 18:0*a*/20:4 | 810.3 | 3.3 ± 0.3 |
| 20:1*a*/18:2 | 812.3 | 1.2 ± 0.1 |
| 20:1*a*/18:1 | 814.3 | 1.2 ± 0.1 |
| 18:0*a*/22:6 | 834.3 | 1.0 ± 0.1 |
| 18:0*a*/22:5 | 836.3 | 1.0 ± 0.1 |
| 18:0*a*/22:4 | 838.3 | 0.6 ± 0.0 |
| 16:0*e*/16:0 | 718.3 | 0.7 ± 0.1 |
| 14:0*e*/20:5 | 738.3 | 1.1 ± 0.2 |
| 16:0*e*/18:2 | 744.3 | 2.6 ± 0.1 |
| 16:0*e*/18:1 | 746.3 | 6.1 ± 0.5 |
| 16:0*e*/18:0 | 748.3 | 1.4 ± 0.3 |
| 16:0*p*/20:5 | 764.3 | 4.2 ± 0.4 |
| 16:0*e*/20:5, 16:0*p*/20:4 | 766.3 | 14.4 ± 0.5 |
| 16:0*e*/20:4 | 768.3 | 8.9 ± 1.0 |
| 18:1*p*/18:1 | 770.3 | 1.0 ± 0.4 |
| 18:0*e*/18:2, 18:1e/18:1 | 772.3 | 1.6 ± 0.2 |
| 18:0*e*/18:1 | 774.3 | 1.5 ± 0.1 |
| 18:0*p*/20:5 | 792.3 | 2.7 ± 0.4 |
| 18:0*p*/20:4 | 794.3 | 4.7 ± 0.1 |
| 18:0*e*/20:4 | 796.3 | 2.3 ± 0.1 |
| 18:1*p*/20:1 | 798.3 | 0.7 ± 0.1 |
| 18:0*p*/20:1 | 800.3 | 1.3 ± 0.1 |
| 20:1*p*/20:5 | 818.3 | 1.5 ± 0.3 |
| 20:1*p*/20:4 | 820.3 | 2.4 ± 0.2 |
| 18:0*e*/22:5 | 822.3 | 1.1 ± 0.1 |
| 18:0*e*/22:4 | 822.3 | 0.6 ± 0.0 |

[a]Phospholipid nomenclature from Reference 1. The letter designates the linkage at the *sn*-1 position in the phospholipid. See Tables 2 and 3 for abbreviations.
[b]The percentage composition was based on the average relative intensities of each molecular ion from the analyses of five separate individuals, mean ± SEM.

platelets and macrophages (23–29%), whereas PC levels (36.3%) were at the lower end of the range seen in these leukocytes (35–46%) (24–26). The higher PE levels in the amebocyte may reflect differences in immune response between invertebrate and vertebrate lymphocytes. Even though the amebocyte can undergo diapedesis, like the macrophage or neutrophil, to reach the site of injury or counter microbial invasion, it is not phagocytic, and it encapsulates foreign bodies by degranulating and releasing clotting factors and antimicrobial substances (8). When compared to the platelet, the amebocyte can plug the site of injury, but unlike the platelet it degranulates initially allowing clot material to fill the site of injury, followed by continued migration of cells to the site of injury, infiltrating and replacing the clot material. These cells then degranulate, elongate, and serve as basis for the formation of new tissue or even carapace (27). The amebocyte PI (6.2%) and PS (9.0%) levels were roughly comparable to the amounts of PI and PS previously reported in platelets and macrophages, 6–8 and 5–11%, respectively. The amount of SPH present in the amebocyte, ~5%, was apparently low in relation to the range of 9–20% SPH detected in mammalian leukocytes (25,26). The amebocyte's CL levels (1.6%) were in a range similar to that reported in human and guinea pig macrophages (1–2.5%) (20,21).

*Analysis of the phospholipid molecular species*. The molecular species composition of the amebocyte phospholipids reported here reflects the key role this hemocyte plays in the immune defenses of this organism. Within the phospholipid classes, a number of overall similarities existed between the molecular species distribution of PS and PI in the amebocyte with vertebrate platelets and macrophages. In the human platelet, the predominant diacyl molecular species of PS are 18:0*a*/20:4 (41%) and 18:0*a*/18:1 (37%) (24), but in the mouse macrophage, PS has three major species 16:0*a*/18:1 (41.1%), 18:0*a*/18:1 (19.8%), and 18:0*a*/20:4 (11.1%) (21). The PS profile of the amebocyte was more similar to the platelet than the macrophage with its predominant PS species of 18:0*a*/20:4 (34%) and 18:0*a*/18:1 (23%). The major PI species of the amebocyte was 18:0*a*/20:4 (52.3%), and the level of this molecular species was comparable to the mouse macrophage 18:1*a*/20:4 levels (59.1%), but was lower than the human platelet that is almost three-quarters 18:0*a*/20:4 (71%). The level of 18:0*a*/20:4 PI is an important indicator of signal transduction processes as this PI species can act as a potential source for the biosynthesis of PI-4,5-bisphosphate. After cellular activation, PI-4,5-bisphosphate is hydrolyzed, and the diacylglycerol portion remains in the plasma membrane inner leaflet while inositol 1,4,5-triphosphate ($IP_3$) is released into the cytosol and stimulates the release of $Ca^{2+}$ from the endoplasmic reticulum, which in turn activates numerous cellular processes (28). The recent detection of a putative $IP_3$ receptor (29) in the *Limulus* amebocyte reinforces the likelihood that there must be a potential source for $IP_3$ in the plasma membrane.

The major species of PE seen in the amebocyte differed markedly from those reported in vertebrate cells. Whereas diacyl species dominated in the PE lipid pools of mammalian leukocytes, ether lipids made up almost three-quarters of the amebocyte PE species. The major PE species reported in mammalian leukocytes were 16:0*a*/20:4, 18:0*a*/20:4, 16:0*a*/18:1, and 16:0*a*/18:2 (21,24,26). The predominant *Limulus* PE species, 20:1*p*/20:5 and 20:1*p*/20:4, were unusual with the eicosene (20:1) group in the *sn*-1 position. One earlier report, in the guinea pig macrophage, discussed small amounts of eicosenoic acid, mixed with 18:3, in ester linkage at the *sn*-1 position, but none in ether linkage (26).

Elevated levels of AA and EPA in the *sn*-2 position in plas-

malogen PE were reported in prior analyses of both *Limulus* nerve tissue and photoreceptor membranes (30,31). These levels of AA and EPA are quite significant to cellular functions. Glaser and Gross (6) demonstrated that the speed of membrane fusion is directly related to the amount of plasmalogen PE molecular species with PUFA in the *sn*-2 position, particularly AA. The rate of membrane fusion, using plasmalogen PE vesicles, is doubled when 20:4 is in the *sn*-2 position rather than 18:1. The predominance of PE plasmalogens species containing AA and EPA is likely to play a significant role in the rapid rate of degranulation of the *Limulus* amebocyte during an immune response to pathogens or external injury. With an open coelom, *Limulus* must be able to react quickly to injury or microbial invasion to preclude systemic contamination, and degranulation promotes the release of clotting and antimicrobial proteins (polyphemusins and antilipopolysaccharide factor) from the cell's granules (8). These levels of polyunsaturation may also affect the membrane fluidity of the amebocyte, as *Limulus* inhabits a broad range of habitats, from the continental shelf (~200 m) to shallow bays (≤5 m) where temperature and salinity ranges are quite extreme (2–3°C/32 ppt to +25°C/13 ppt) (32).

The PC species observed in the amebocyte, as with the PS and PI pools, were similar to those seen in mammalian leukocytes. The diacyl PC species that predominated in mammalian leukocytes were 16:0*a*/18:1, 16:0*a*/18:2, 16:0*a*/16:0, and 16:0*e*/20:4 (21,24,26). In *Limulus*, the pattern was similar, except the major species were alkylether and plasmalogens, 16:0*e*/20:5, 16:0*p*/20:4, 16:0*e*/20:4, and 16:0*e*/18:1. As reported for the PE species, high levels of AA and EPA were also detected in the PC fractions of *Limulus* nerve tissue (30%) and photoreceptor membranes (42%) (30,31).

In the present study of the amebocytes, the detection of 16:0*e*/20:4 as major species in the PC phospholipid pool is significant, as it is considered to be a precursor to platelet-activating factor (PAF) (1-*O*-alkyl-2-acetyl-PC) (33). PAF-like lipids were detected in as many as 30 species of invertebrates (2,3), but this is the first documentation of the presence of a specific precursor to PAF in an invertebrate hemocyte. PAF is involved in both signaling and adhesive events between proinflammatory cells and vascular tissues (34). The presence of this potential precursor to PAF is a further indication that signal transduction pathways seen in modern vertebrate cells may also occur in this primitive invertebrate blood cell.

## ACKNOWLEDGMENTS

The research presented in this paper is funded, in part, by a grant from the National Sea Grant College Program, National Oceanographic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA-36RG0537, project number R/MP-71 through the California Sea Grant College System, and in part by the California State Resources Agency. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its subagencies. Jennifer MacPherson's support is funded through the Sea Grant Traineeship Program.

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[Received January 6, 1998, and in final revised form May 27, 1998; revision accepted August 10, 1998]