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UNIVERSITY OF CALIFORNIA

Santa Barbara

Studies on the Mechanism of Eicosanoid Biosynthesis in the Primitive Arthropod, *Limulus polyphemus*

A Dissertation submitted in partial satisfaction

of the requirements for the degree of

Doctor of Philosophy

in

Biological Sciences

by

Jennifer Catriona MacPherson

Committee in Charge:

Professor Robert S. Jacobs, Chairman
Professor Robert K. Trench
Professor Kathleen Foltz

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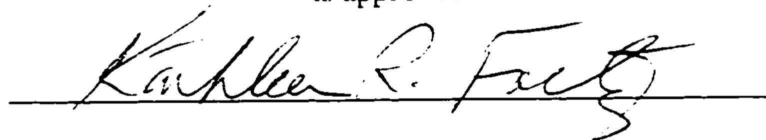

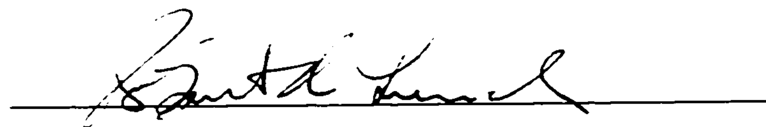

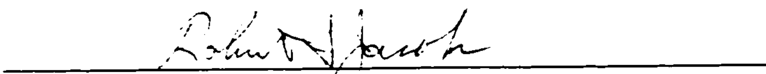

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DEDICATION

Without a doubt. I have to dedicate this thesis to my mother Barbara MacPherson. Not only did she bring me into this world. but she knew when to keep pushing me when I had doubts. Her unflagging love and support have kept me going.

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ABSTRACT

Studies on the Mechanism of Eicosanoid Biosynthesis

in the Primative Arthropod, *Limulus polyphemus*

by

Jennifer Catriona MacPherson

The studies presented are designed to increase our understanding of the biochemical pathways involved in inflammation using a primordial model. *Limulus polyphmus* is an ancient marine arthropod which has retained practically the same bodily form for 300 million years. *Limulus* has a single circulating blood cell, the granular amebocyte. This hemocyte was studied to determine if it could produce eicosnaoids involved in the inflammatory process, like those seen in mammalian cells, when stimulated with calcium ionophore and the fatty acid precursor, arachidonic acid. The studies revealed that the cell is capable of producing eicosanoids, and the major metabolite was 8-hydroxyeicosatetraenoic acid (8-HETE). HETEs are known to be involved in both inflammatory and reproductive processes of invertebrates and mammals.

The lipid composition of the amebocyte was then examined to determine the possible pools of eicosanoid precursors in the hemocytes. This analysis revealed large levels of twenty carbon polyunsaturated fatty acids, especially arachidonic and eicosapentaenoic acids, are present in the amebocyte membranes. The phospholipid class analysis revealed that phosphatidylethanolamine levels (42.2%) were followed by phosphatidyicholine (36.3%), phosphatidylserine (9.0%), phosphatidylinositol (6.2%) and sphingomyelin (4.6%). Cardiolipin (1.6%) was also present, as well as

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trace amounts of lysophosphatidylcholine. The phosphatidylethanolamines contained plasmalogens (62%) and alkylacyl phospholipids (27%), but little diacyl phospholipid (11%). The phosphatidylcholines were diacyl (39%), alkylacyl (35%) and plasmalogen (26%). PI and PS were predominantly diacyl. Especially interesting was the presence of 16:0e/20:4 phosphatidylcholine, a precursor to platelet activating factor (PAF), and this is the first documentation of a specific PAF precursor in an invertebrate hemocyte.

A putative phospholipase A_2 (PLA_2) was partially purified by three chromatographic steps and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and ESI-MS. The band reactive to polyclonal recombinant human PLA_2 in the Western blot was 18.5 kDa protein in the ESI-MS analysis. A partial sequence revealed that the protein was previously described, though with alternative activity (Fujii *et al.* 1992). The protein reacted in a well-established *E. coli* PLA_2 assay and demonstrated specificity with no general lipase activity. The protein's activity was inhibited in a dose-dependent fashion by the irreversible inhibitor, manoalide, as well as by BPB which binds to histidine at the active site of PLA_2s. The protein did not lose activity after heating alone, but activity was abolished after heating with ßME. The characterization of this putative PLA_2 from *Limulus* will contribute to our understanding of the evolution of the pathways involved in inflammation, as well as possible alternate functions for this protein in invertebrates.

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ABBREVIATIONS

AA	Arachidonic acid
BPB	bromophenacyl bromide
CI	Chemical ionization
CL	Cardiolipin
CO	Cyclooxygenase
DMA	Dimethylacetal
EI	Electron impact
EPA	Eicosapentaenoic acid
ESI-MS	Electrospray ionization - mass spectrometry
FAB	Fast atom bombardment
FAME	Fatty acid methyl ester
GC-MS	Gas chromatography - mass spectrometry
GPLR	G-protein linked receptor
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HETrE	Hydroxyeicosatrienoic acid
HHTrE	Hydroxyheptadecatrienoic acid
HOTrE	Hydroxyoctadecatrienoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High pressure liquid chromatography
HpODE	Hydroperoxyoctadienoic acid
IP_3	Inositol-1,4,5-triphosphate
KETE	Ketoeicosatetraenoic acid
LO	Lipoxygenase
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
MLD	Manoalide
MO	Monooxygenase
PAF	Platelet activating factor
PC	Phosphatidylcholine

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PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol-4.5-bisphosphate
PLA_2	Phospholipase A_2
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
$rhPLA_2$	Recombinant human phospholipase A2
RP-HPLC	Reverse phase - high pressure liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIMS	Secondary ion mass spectrometry
SIR	Selected ion recording
SP-HPLC	Straight phase - high pressure liquid chromatograpy
SPH	Sphingomyelin
TPA	Tetradecanoylphorbol-13-acetate

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1.0 Introduction

1.1. *Limulus polyphemus*

Horseshoe crabs are marine arthropods of the Class Merostomata. Though initially described by zoologists as crabs, these organisms are more closely related to arachnids than crustaceans (Figure 1). Four extant species of horseshoe crabs are known worldwide, *Limulus polyphemus*, *Tachypleus tridentatus*, *T. gigas* and *Carcinoscorpious rotundacauda*. *Limulus polyphemus* is found along the eastern shores of North and Central America ranging from Maine to the Yucatan Peninsula. The remaining species, *T. tridentatus*, *T. gigas* and *C. rotundacauda*, are spread throughout Southeast Asia from Japan to the eastern border of India. The American and Asian species are both thought to descend from a common ancestor, *Mesolimulus* sp., which lived approximately 100 million years ago. Horseshoe crabs have retained the same body form for practically the last 300 million years and are considered a living fossil. The endurance of this species is due to its remarkable versatility. They are capable of withstanding great variations in temperature, salinity and even prolonged exposure to the air (Shuster 1990, 1982) which enables them to survive a lengthy development period. *Limulus* must undergo sixteen to seventeen molting instar stages over a nine to ten year period before reaching maturity (Sekiguchi and Yamasaki 1988).

Horseshoe crabs have served as research models as far back as the 1880's. Early investigators examined their morphology, nervous system and blood circulation patterns. More recently, *Limulus* has emerged as an important model for comparative physiological and immunological studies due to its size (up to ten pounds), longevity

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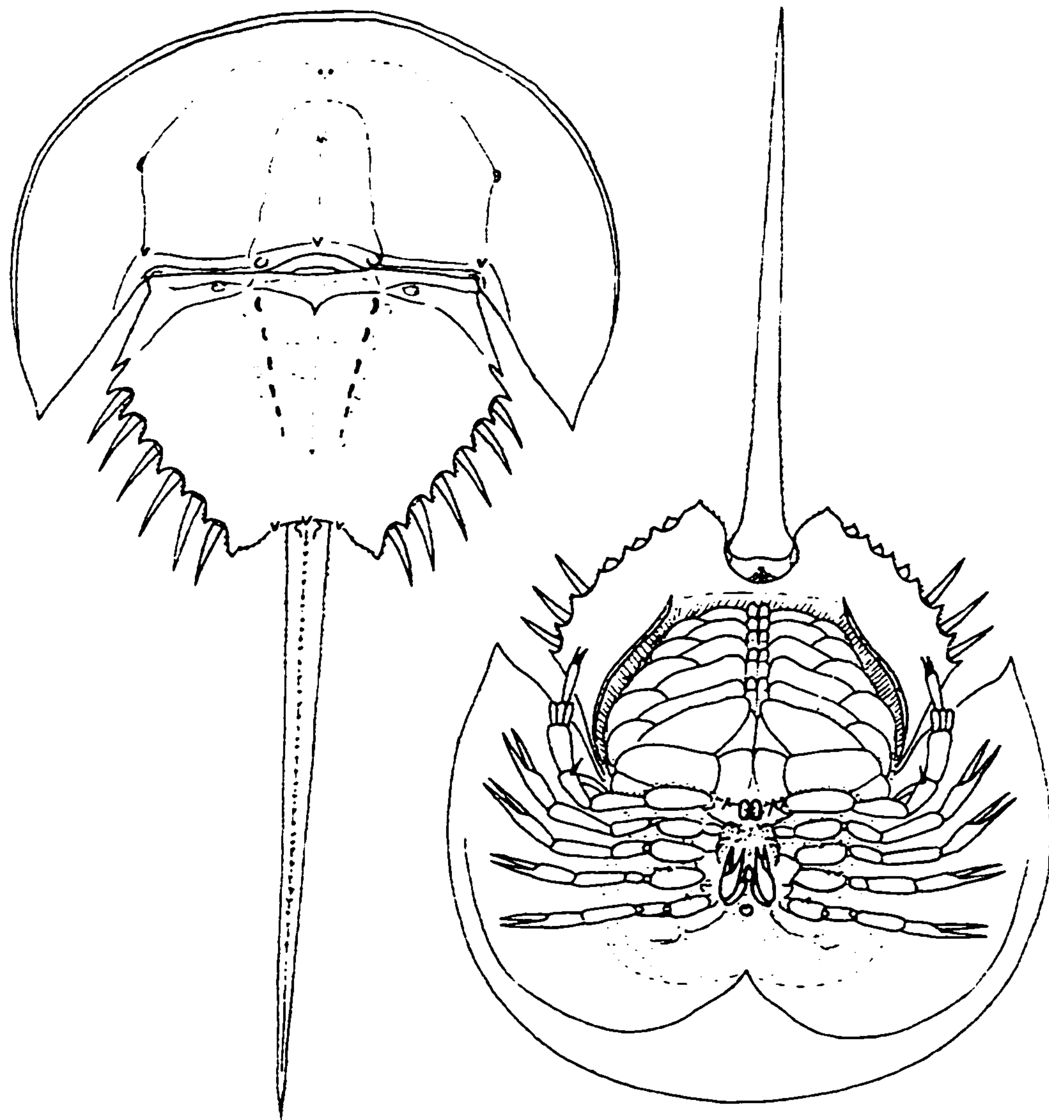

Figure 1. General morphology of a female horseshoe crab. (adapted from Sekiguchi and Yamasaki 1988)

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(about 9-14 years) and the fact that large amounts of blood can be drawn (100-150 ml) without killing the animal (Armstrong 1985a). Presently, studies are focused around the phototransduction pathways in their photoreceptors, as well as in-depth work on the signal transduction and enzymatic pathways involved in endotoxin-induced aggregation, degranulation, and coagulation of their blood cells. The significance of studies involving *Limulus* hemocytes, termed amebocytes, is reinforced by their classification as 'primordial immunocytes'. It has been hypothesized that these blood cells share some of the same functions seen in platelets and macrophages, as well as B- and T-lymphocytes (Gupta 1991). The similarity in basic cellular functions between *Limulus* amebocytes and these vertebrate hemocytes makes the amebocyte a unique choice for studying the origins of enzymes and metabolites involved in the inflammatory process.

1.2 Invertebrate Immunology

Two mechanisms are associated with an immune response: innate and acquired. These mechanisms operate through the combination of humoral factors, found in the blood plasma, and the cell-mediated actions of the hemocytes. According to established immunological dogma, invertebrate immune reactions function in an innate, or natural, manner (Bigger 1988). In essence, the immune response automatically reacts indiscriminately to the presence of foreign materials and is not enhanced by previous exposure to these substances (Weir and Stewart 1993). Innate immunity can be implemented by mechanical barriers, surface secretions, the normal bacterial flora, or humoral defense mechanisms which include lectins, acute phase proteins and interferons. Humoral factors are normally present at very low

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concentrations in the blood plasma. Their levels rise quite rapidly when an organism is challenged by infection. In contrast, the vertebrate immune system can respond in either an innate or acquired manner. Acquired, or adaptive, reactions are triggered by a memory process that is initiated by previous exposure to foreign material (Weir and Stewart 1993). As with innate responses, acquired immunity can be mediated through several pathways including, but not exclusively, humoral reactions, creation of circulating antibodies, cell-mediated phagocytosis and encapsulation.

In both invertebrates and vertebrates, the immune system can be challenged by infections due to pathogenic microbes and cancerous growths throughout their life cycle (Marchalonis and Schulter 1990). The humoral and cell-mediated defenses work synergistically with each other to counter foreign invasion (Weir and Stewart 1993). The coordinated response between these two systems can sometimes make it difficult to clearly distinguish the cell-mediated responses. There are invertebrate immune factors which display gross functional similarities with compounds involved in the vertebrate complement, blood coagulation and humoral response; but it should not be assumed that all immune response mechanisms found in vertebrates will necessarily be present in invertebrates. For example, invertebrate blood cells have not been shown to express recognition proteins like the major histocompatibility complex (MHC) or to produce circulating antibodies. Invertebrate hemocytes have surface molecules, usually in the form of carbohydrates or glycoproteins, which recognize molecules associated with the cell surface of pathogenic organisms. In *Limulus*, it has been demonstrated that its hemocytes indiscriminately recognize the lipid A portion of lipopolysaccharide from the cell membranes of any gram-negative bacteria

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(Iwanaga *et al.* 1992). Of those factors which exhibit structural features similar to those seen in vertebrate systems, most display limited sequence similarity.

The mechanisms involved in the humoral and cell-mediated defense responses of *Limulus* have likely developed in response to the challenges it faces in its environment. Horseshoe crabs live at the benthic interface in the deep ocean and estuaries where they are exposed to large numbers of bacteria (Pistole and Graf 1986), viruses and, in more recent times, anthropomorphic pollutants from farming and industrial outflow. *Limulus*' immune responses must combat the onslaught of ectocommensals, parasites and microbes which they encounter in their habitat (Figure 2) (Iwanaga *et al.* 1992, Bang 1979, 1956). Frequent molting during the first five to seven years is a tactic which allows juveniles to rid themselves of most external pests. Horseshoe crabs can also exude a glycoprotein from their hypodermal glands through channels in the carapace. This secretion presents a physical barrier to bacteria and has the ability to agglutinate them, but it is not bacteriacidal (Stanger and Redmond 1975). Therefore, *Limulus*' immune system, though not as sophisticated as that seen in vertebrates, provides an effective means for their survival in a harsh environment.

Research on the humoral immune system of *Limulus* has revealed a number of important compounds. These include the alpha$_2$-macroglobulins which function as serine protease inhibitors (Armstrong and Quigley 1991), C-reactive proteins (Ying *et al.* 1992), small molecular weight antimicrobial factors (Yeo *et al.* 1993) and lectins. The alpha$_2$-macroglobulins are considered the first line of attack for humoral defense (Armstrong 1991) because proteases convert a variety of proteins from their inactive to active forms. Proteases are involved in the invasion and pathogenesis of bacteria, molds and multicellular parasites (Armstrong and Quigley 1991). Furthermore, the

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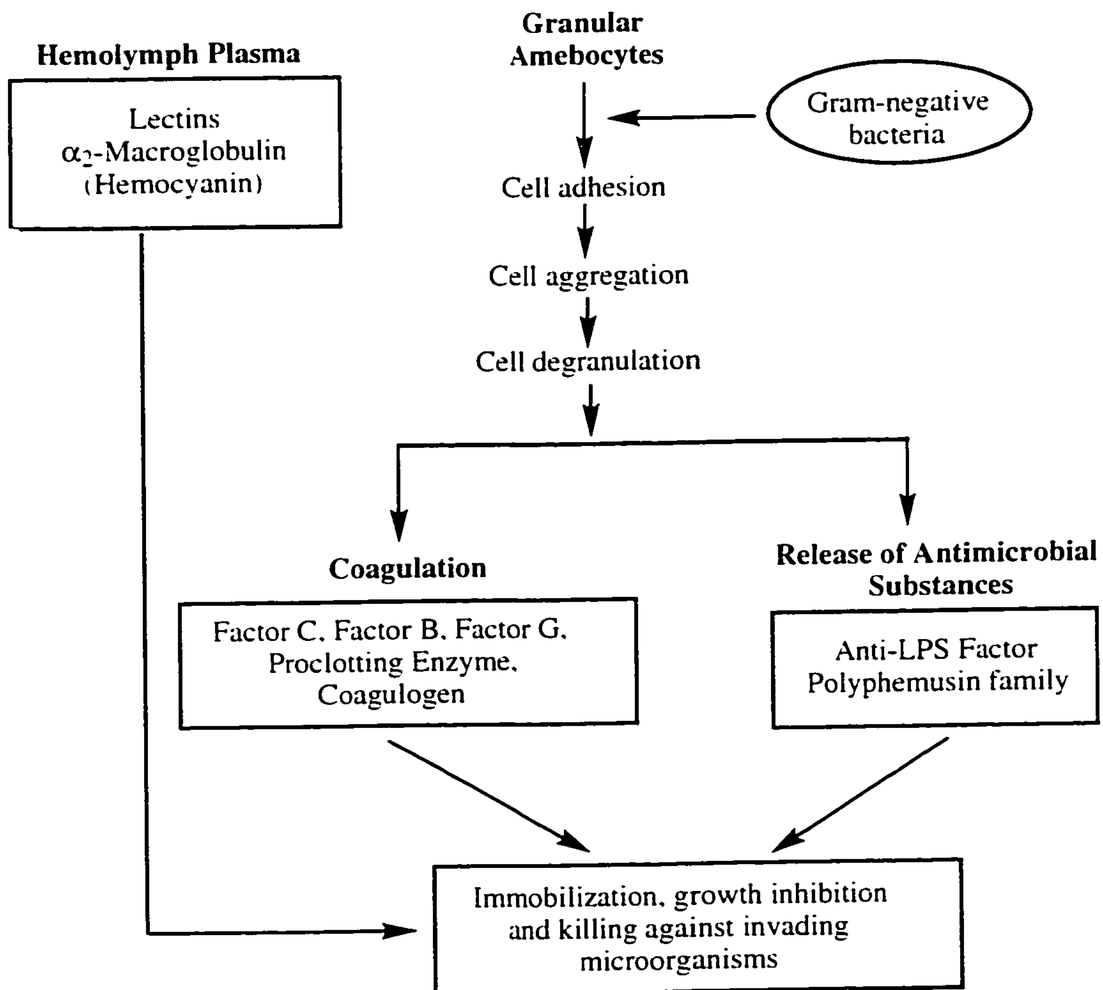

Figure 2. Cellular and humoral defense systems in *Limulus*. (adapted from Iwanaga *et al.* 1992.)

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horseshoe crab blood coagulation pathway involves the sequential activation of serine proteases. The uncontrolled activation of the coagulation cascade, in response to a bacterial infection, can also pose a potential threat to the horseshoe crab. The continuous formation of clot material will eventually fill the carapace effectively suffocating the animal. Therefore, in *Limulus*, alpha$_2$-macroglobulins are able to regulate and inactivate proteases of both endogenous and exogenous origins.

Lectins have been detected in both the hemolymph and on the amebocytes of *Limulus* but their function is not completely understood. In invertebrates, they have been linked to cellular aggregation, opsinization, inhibition of cell growth, and phagocytosis, as well as acting as cell surface receptors and acute phase proteins (Ying *et al.* 1992, Gupta 1991, Vasta and Marchalonis 1984). In *Limulus*, lectins are most likely involved in aggregating bacterial cells. The ability to agglutinate foreign cells enables the horseshoe crab, in conjunction with blood coagulation, to localize a microbial invasion and prevent it from spreading.

Further studies on invertebrate defense systems have examined the roles of cell-mediated phagocytosis, encapsulation of pathogens or parasites, degranulation followed by the release of cytosolic factors and expression of humoral compounds (Harvell 1990, Armstrong and Quigley 1991). As it has only one type of circulating hemocyte, studies concerning the cell-mediated response in *Limulus* have centered around these blood cells. During the last twenty years, the bulk of the immunological research on these hemocytes has focused on isolating and identifying the compounds involved in blood clotting and elucidating their molecular mechanism of action. Only recently has work has begun to appear about the signal transduction pathways possibly involved in this process (Solon *et al.* 1997, 1996).

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1.3. Cell-Mediated Immune Response in *Limulus* Amebocytes

Three types of blood cells have been documented to exist in horseshoe crabs: the cyanocyte, granular amebocyte and plasmocyte. The cyanocytes originate as cyanoblasts which are found freely circulating during the embryological stages, but within the intermolt adult they are found solely in the vascular spaces of the ocular ganglion (Armstrong 1991). The cyanoblast begins to produce hemocyanin crystals singly and in arrays within the cytoplasm until the cell is almost completely filled with the crystal and at this point it is termed a cyanocyte. At this stage the cell ruptures and releases the hemocyanin crystal arrays into circulation. The arrays disperse as the ends of the columnar arrays degrade into the hemolymph (Farenbach 1970). The isolation of the cyanocyte from circulation indicates that it is unlikely to interact or function with presently described aspects of this animals' immune system.

The remaining cells, plasmocytes and granulocytes, are both circulating hemocytes. Plasmocytes represent about three percent of the hemocyte population in *Tachypleus* sp. but are absent in *Limulus* (Jakobsen and Suhr-Jessen 1990, Armstrong 1985a). The granulocyte, or granular amebocyte, consitutes the majority of the hemocytes (~97%) in *Tachypleus* sp. and is considered the sole blood cell type present in *Limulus*. The cell was named by Loeb (1920) for its ameboid-like movements observed under the microscope after degranulation. Amebocytes are round or ellipsoid, 10-20 μm long in length and the nucleus is obscured by the presence of many refractile granules (0.5-2 μm diameter) (Figure 3) (Iwanaga 1993, Armstrong 1985a). The plasmocyte is comparable in size to the amebocyte but its granules are smaller (about 0.6 μm) and the intracellular contents are quite different.

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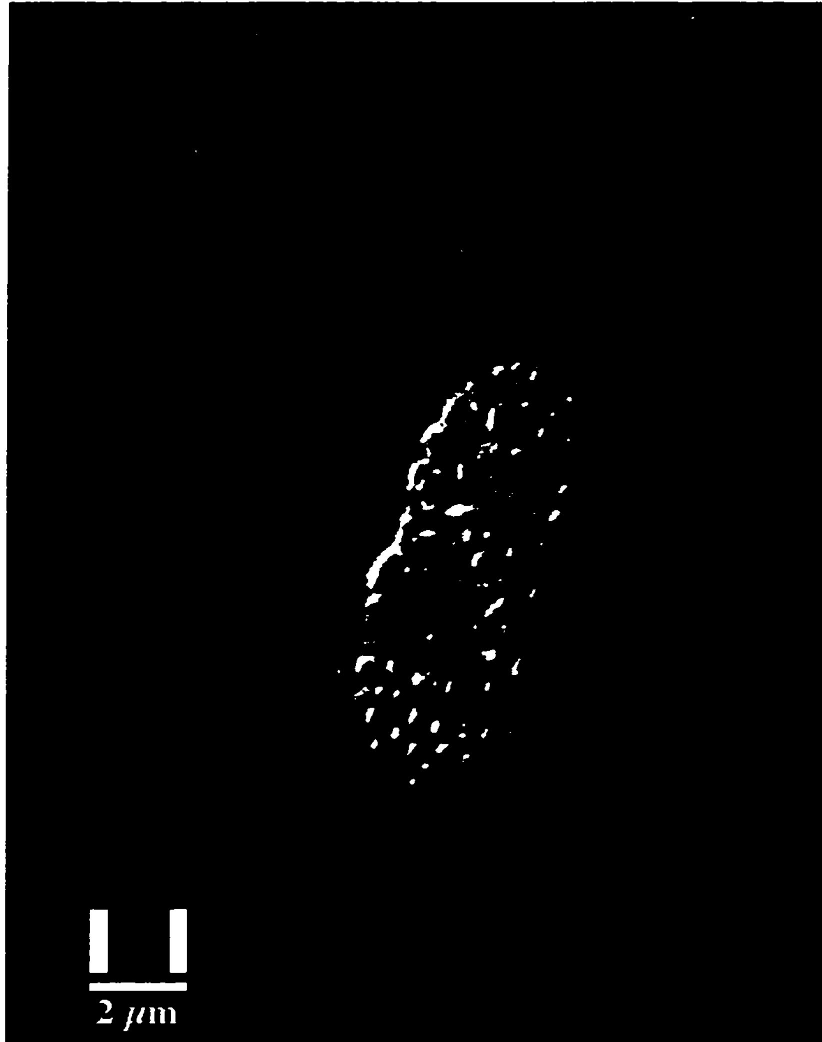

Figure 3. A Nomarski differential interference contrast photograph of a *Limulus* amebocyte (100X) using a BX60 Olympus microscope.

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A healthy horseshoe crab averages between 2 to 6 x 10^7 cells/ml. Yeager and Tauber (1935) found that in seventy-one separate counts, from twenty-seven adults and eight juveniles, that less than one cell per two thousand counted was undergoing any mitotic activity. More recently, Chen *et al.* (1989) were unable to detect DNA synthesis occurring in amebocytes maintained in culture for a week. Therefore, these cells could be in the equivalent of the mammalian cell's state of terminal differentiation.

The most pronounced immune response of the amebocyte is degranulation and the subsequent formation of coagulin gel in the presence of bacterial lipopolysaccharide (LPS). A LPS-binding receptor in the plasma membrane of the amebocyte detects the presence of endotoxin in the blood. As previously stated, the receptor has been found to react specifically with the lipid A portion of endotoxin (Iwanaga *et al.* 1992). Several LPS-binding proteins (12, 50, 80 and 82 kDa) have been isolated and characterized from the cell membranes of *Limulus* amebocytes (Liang *et al.* 1981,1980, Minetti *et al.* 1991, Roth and Tobias 1993). The 82 kDa protein isolated by Liang *et al.* (1981) was shown to activate a phosphodiesterase in a Ca^{2+}-dependent manner, as well as exhibiting calmodulin-like activity. Since calmodulin (CaM) is known to regulate cytosolic calcium levels, the stimulation of CaM-like activity could also result in an increased level of intracellular calcium stimulating degranulation. It has been postulated that the LPS-binding protein functions as a G-protein-linked receptor (GPLR) (Liang *et al.* 1981, Armstrong 1991). Solon *et al.* (1996) tested the hypothesis that LPS-induced exocytosis is mediated by a GPLR using a series of inhibitors and activators. Amebocytes were pretreated with GPLR modulators (cholera and pertussis toxins), a protein kinase C inhibitor (chelerythrine), a tryosine kinase inhibitor (herbimycin) or a phospholipase

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C inhibitor (U73122). The cells were then stimulated and in cases of pretreatment with GPLR or phospholipase C inhibitors the cells did not degranulate in the presence of LPS. The protein kinase C and tryosine kinase inhibitors, though, did not block degranulation. This indicates that degranulation may be activated through a GPLR mediated pathway. Unfortunately, the cellular response was ranked only as a positive (50% cells undergo 100% degranulation) or negative (<50% cells undergo 100% degranulation) reaction. Nonparametric data are difficult to interpret because they are often used when only weak assumptions can be made about the distribution of the data (Steel and Torrie 1980). Other difficulties lie in the experimental design. The assays were run for an hour and during this time period amebocytes will begin to spontaneously degranulate regardless of their treatment (Armstrong 1985a). Also, the concentration of inhibitors used to block cellular respose were excessively high (Dr. K. Foltz, pers. comm.). Another portion of Solon *et al.*'s (1996) studies tried to demonstrate that ameboctyes could be induced to degranulate through microinjection of inositol trisphosphate (IP_3), Ca^{2+} or Mg^{2+} alone or in mixtures. The data presented here was particularly weak as the authors placed too much emphasis on the importance of Mg^{2+}, yet their own data shows uncontrolled degranulation levels of 90-100%, independent of the concentration of Mg^{2+} injected (1, 2, 5, 9.2 and 46 mM). The Ca^{2+} injections, on the other hand, demonstrated a clear physiological response with degranulation increasing over the concentration gradient (1, 2, 5 and 10 mM). Also, in the presence of cholera and pertussis toxins, Mg^{2+} precipitated degranulation, even when LPS did not. Therefore, though their data indicates that induction of degranulation of the amebocytes is mediated by G-protein receptor linked to a phospholipase C, it is not strong enough to disprove the possibility that more than one pathway exists.

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The components involved in the clotting reaction are stored in two types of intracellular granules, termed large (L) and small (S). The L granules contain the four serine protease zymogens, Factor C, Factor B, the proclotting enzyme and Factor G, which are directly involved in clot formation (Figure 4). These granules also contain coagulogen, the Anti-LPS factor, transglutaminase substrates, some lectins and several unknown proteins. Polyphemusins, small antimicrobial proteins, are found in the S granules. Six other proteins have also been seen in S granule preparations, but they remain unidentified (Iwanaga *et al* 1992). The exocytosis of the S granules follows the L granules (Toh *et al.* 1991). The release of these granules initiates the formation of a coagulin gel clot which immobilizes the microbes. The encapsulated microbes are then killed by a combination of the polyphemusins and humoral factors (Yeo *et al.* 1993, Ohta 1992, Niwa *et al.* 1988). This cell-mediated immune response is *Limulus*' primary line of defense.

Prior studies on the cell-mediated immune response of amebocytes have emphasized circulating humoral factors and compounds stored in the cells' granules. No consideration has been given to the possible presence of low molecular weight secreted lipid metabolites known to be involved in vertebrate cell-mediated immune response. These metabolites, termed eicosanoids, are generated during an inflammatory response and some of these compounds are known to have chemotactic properties. Eicosanoids have been documented in invertebrates (Stanley-Samuelson 1991) and a study by Bursey (1977) suggests the involvement of "mediators" during injury to *Limulus*. Bursey mimicked injury to the animals by perforating the opisthoma, the central body segment, and then histologically monitoring changes at the site of injury. He found that amebocytes migrated through connective tissue to the site of injury within 24 hours to form a layer surrounding the wound area.

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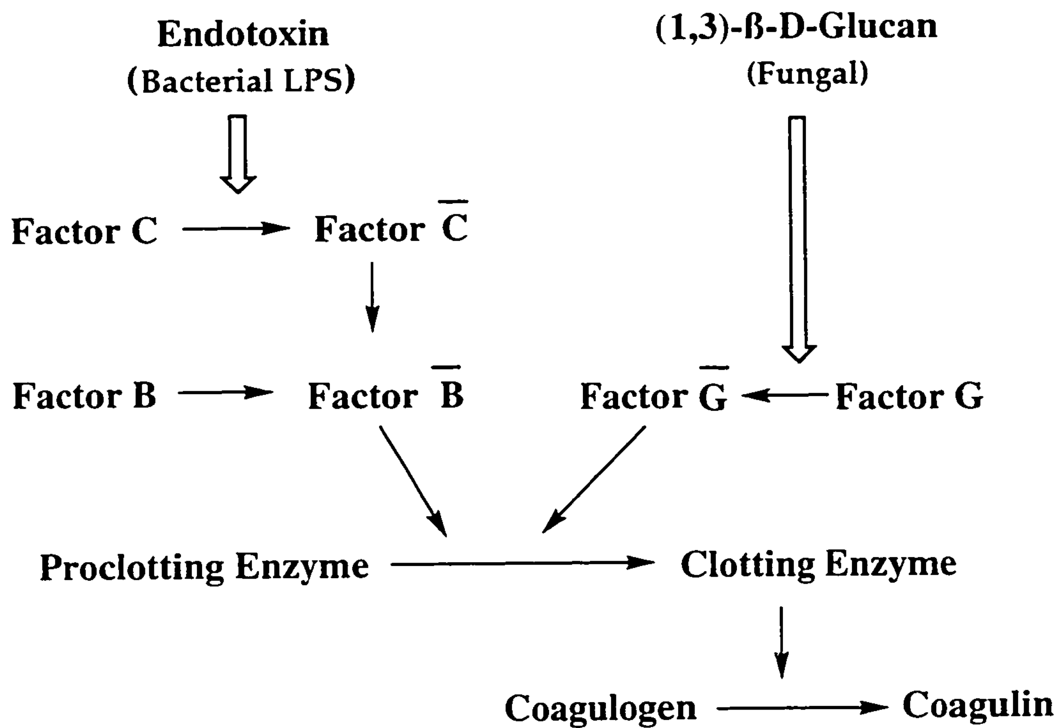

Figure 4. Cascade of serine proteases leading to clot formation after cellular degranulation due to immunological challenge by foreign pathogens.

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Hemocytes continued to migrate to the site of injury for an additional four days. The protracted movement of cells to the site of injury is indicative of the release of chemotactic factors, possibly lipid metabolites. Hence, studies on the production of lipid metabolites during degranulation would advance our understanding of the processes involved in the immune response of *Limulus* amebocytes

1.4 Eicosanoid Biology

The enzymatic pathways involved in the biosynthesis of eicosanoids have been intensely studied in mammalian blood cells. After little more than a decade of study, researchers are only beginning to define the processes controlling their production in invertebrates (Stanley-Samuelson 1991, 1987). Though the presence of eicosanoids has been documented in over a hundred invertebrates, both terrestrial and marine, not many studies have attempted to link these metabolites to their immune functions. Eicosanoids are known to help mediate mammalian immune response during an acute inflammatory response and Stanley-Samuelson (1991) has hypothesized that a crucial early step in cell-mediated immune response to bacterial infections (i.e. stress) in insects and other invertebrates is the biosynthesis of eicosanoids. To understand the biosynthetic pathways involved in eicosanoid formation, as well as the physiological roles these molecules may play, it is necessary to determine how production is stimulated (DePetrocellis and DiMarzo, 1994). The investigation of biosynthetic pathways in the hemocytes of marine organisms is difficult because of the lack of methods for routinely maintaining these cells in culture outside of the animal. To date, the majority of studies on eicosanoids in invertebrates

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have been carried out by extracting the compounds from whole organisms or individual organs from the body.

The first evidence of eicosanoids in marine organisms came from the discovery of 15-epi-prostaglandin A_2 and its methyl ester acetate in the gorgonian, *Plexaura homomalla* (Weinheimer and Spraggins 1969). A synthetic pathway for its formation was proposed during prostanoid studies on the soft coral *Clavularia virdis* (Corey *et al.* 1987). This synthesis began with arachidonic acid (AA) being converted to 8-(R)-hydroperoxyeicosatetraenoic acid (HPETE) and then, via an allene oxide followed by an oxido-pentadienyl cation, to a prostanoid form. Bundy *et al.* (1986) have already demonstrated the presence of 8-R-lipoxygenase in gorgonians and the presence of an allene oxidase synthase, in marine organisms, was later confirmed during unrelated studies on eicosanoids found in starfish oocytes (Brash *et al.* 1991). This pathway differs greatly from the prostaglandin endoperoxidase mediated cyclooxygenase (CO) or monooxygenase (MO) pathways seen in mammalian systems (Figure 5).

Eicosanoid production in invertebrates has been linked to reproduction, development, epithelial transport, synaptic function and hemocytic defense responses. In hydroids, DiMarzo *et al.* (1993) detected the production of hydroxyeicosatetraenoic acids (HETEs) during tentacle budding. Meijer *et al.* (1986) described the production of 8-(R)-HETE, which acts like a hormone in mediating oocyte meiosis reinitiation, in four genera of starfish (*Asterias rubens, Marthasterias glacialis, Orthasterias koehleri* and *Evasterias troschelii.*). Scheul (1984) has further suggested that cyclooxygenase and lipoxygenase (LO) metabolites could modulate gamete interaction and aid in preventing polyspermy. In marine molluscs, prostaglandins have been implicated in both receptor mediated ion regulation (Freas and Grollman 1981), and in the induction

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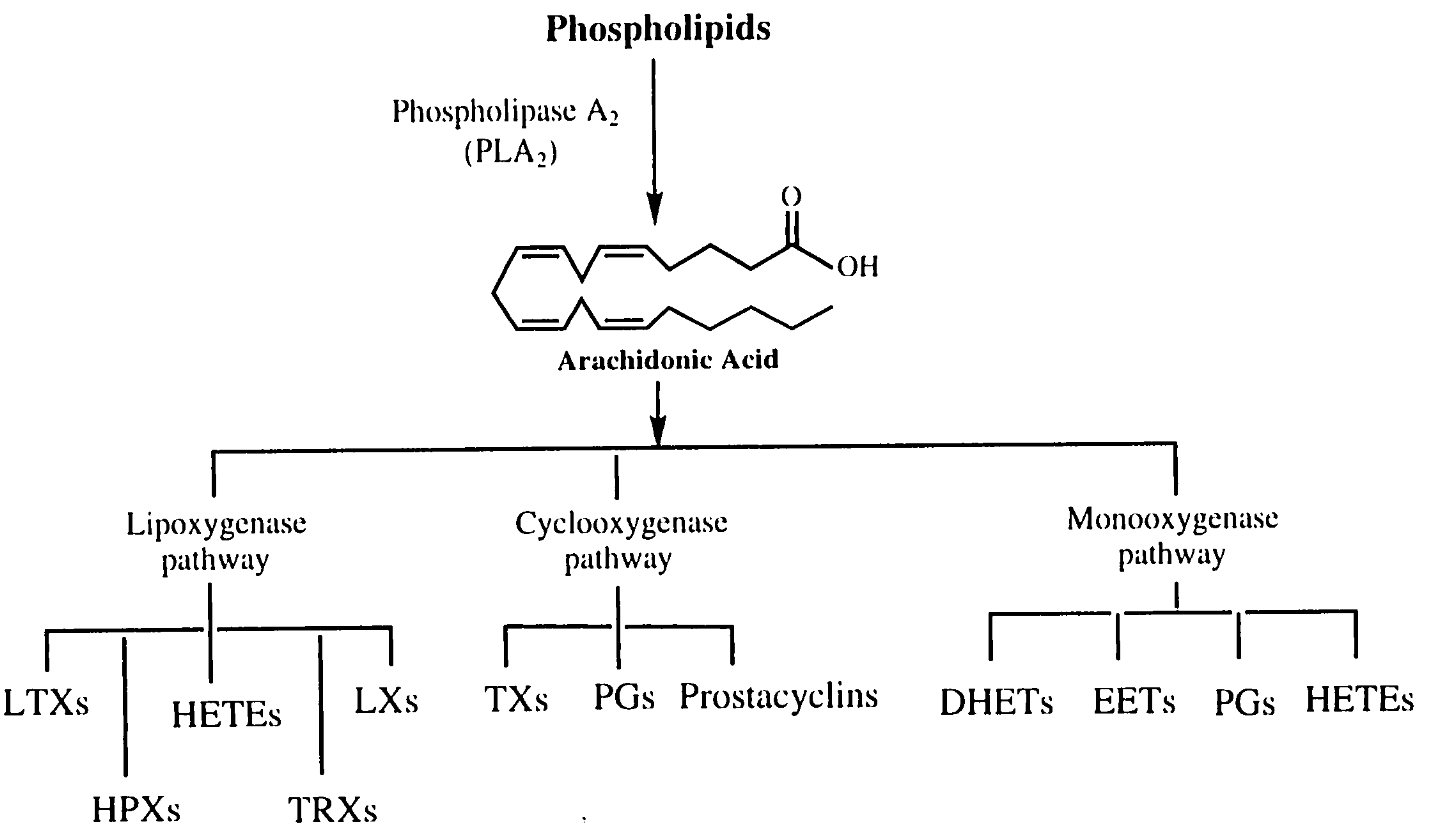

Figure 5. Generalized diagram of arachidonic acid biosynthetic pathways. (Abbrev: LTXs, leukotrienes; HPXs, hepoxilins; HETEs, hydroxyeicosatetraenoic acids; TRXs, trioxylins; LXs, lipoxins; TXs, thromboxanes; PGs, prostaglandins; DHET, dihydroxyeicosatrienoic acids; EETs epoxyeicosatrienoic acids)

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of spawning (Morse *et al.* 1977). Piomelli (1991) has detected 12-HPETE, hepoxilins and ketoeicosatetraeicosanoic acids (KETEs) in the nervous system of *Aplysia californica* and believes they are involved in neuronal intracellular signaling. In addition, a unique tri-HETE and 8-hydroxyeicosapentaenoic acid, which act as hatching factors, have been found in barnacles (Hill *et al.* 1988, Clare *et al.* 1986,olland *et al.* 1985). The biosynthesis of prostaglandins and HETEs in terrestrial arthropods has been linked to the animals' ability to clear bacterial infections and is therefore inferred to be involved in the animals defense response (Gadelhak *et al.* 1995).

Despite the fact that many eicosanoid metabolites have been detected in marine organisms, the enzymatic pathways involved in their production have not been clearly defined. The biosynthesis of eicosanoids is believed to be regulated by the cleavage of precursor fatty acids, such as arachidonic acid, from the phospholipid bilayer (Waite 1987). Though there are alternate enzymatic pathways involving diacylglycerols, the main enzymatic pathway responsible for the release of the precursor fatty acids from phospholipids is phospholipase A_2 (PLA_2).

1.5 Phospholipases A_2

Phospholipases A_2 are signal transduction enzymes which belong to a growing superfamily (Dennis 1997). Within cells, these enzymes can be located in the cytosol, translocate to the membrane or are secreted after stimulation. The position of the fatty acids within the phospholipid is important (Figure 6). On the glycerol backbone of the phospholipid, there is a tendency for the carbon chain on the first carbon to be saturated and the carbon chain on the second carbon to be

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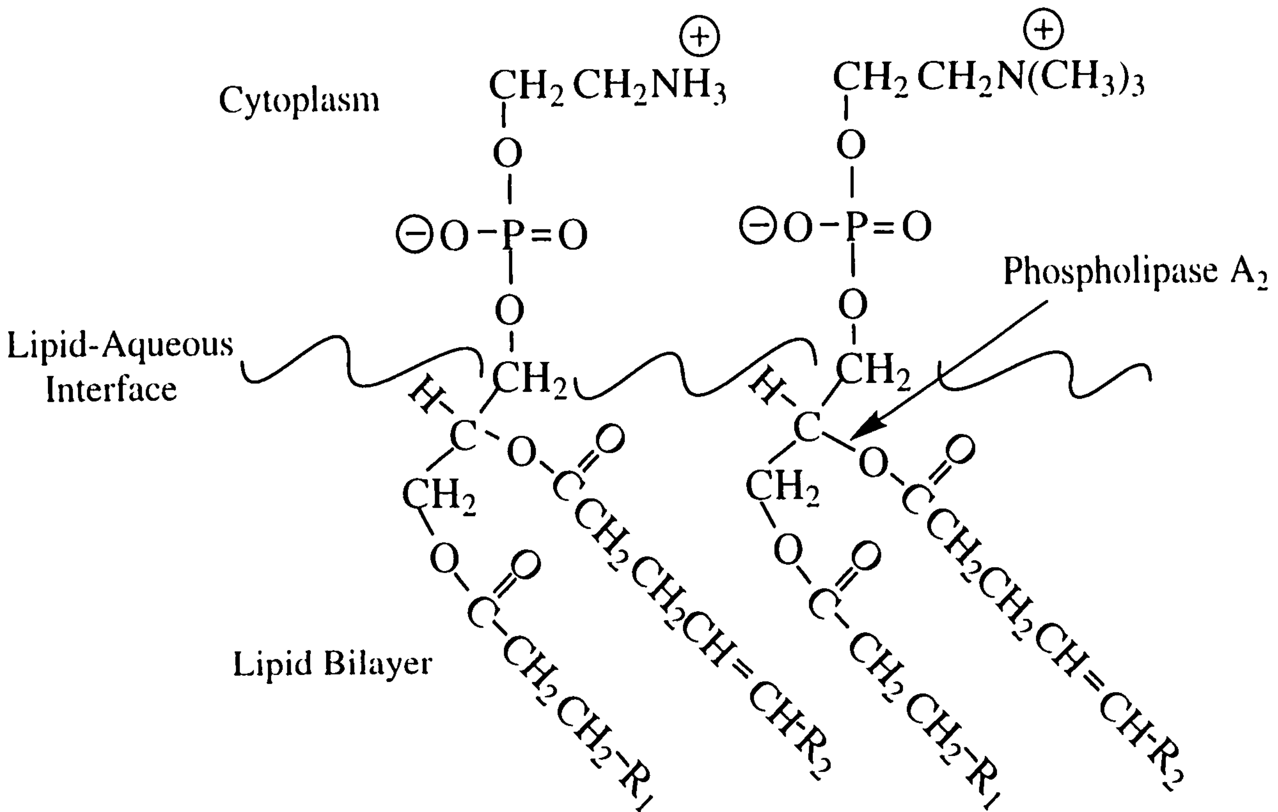

Figure 6. Phospholipase A_2 cleavage of a fatty acid from the second carbon in the glycerol backbone. The proximity of the fatty acid to the lipid-aqueous interface allows better access of the enzyme to the ester linkage.

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unsaturated (Kates 1986) while the head group is linked to the third carbon. The head group is in the aqueous cytosol or extracellular environment. which positions the unsaturated fatty acid at the second carbon near the interface of the lipid bilayer and the hydrophilic environment. making it readily accessible to a PLA_2 (Waite 1987).

In Dennis' (1997) recent review he classifies nine major groups of PLA_2 with two of these groups (I. II) containing several subtypes. The majority of the PLA_2s (groups I. II. III. V and IX) are small. secreted enzymes which average 13-18 kDa. The other groups (IV. VI. VII and VIII) are larger (29. 45 and 85 kDa) and mainly cytosolic. Many of the secreted forms are venoms from snakes. insects and lizards. However. several are present in mammalian macrophages. platelets and blood plasma as well. The larger cytosolic enzymes have been detected in macrophages. platelets. CHO cells. rat kidney. canine myocardium and bovine brain. The smaller enzymes exhibit a millimolar dependency for $[Ca^{2+}]$ levels. while the larger cytosolic forms require less than micromolar levels. if any at all. It should also be noted that some of the PLA_2s demonstrate more than one type of enyzmatic activity. For example. the group IV. 85 kDa PLA_2 also possesses lysophospholipase and transacylase activity (Sharp *et al.* 1994). Snake venoms have also been shown to display proclotting and aggregation activities (Ouyang *et al.* 1992). Considering the amount of work done in this field. it is interesting that to date only three reports of PLA_2s in invertebrates have been published, two in insects (Uscian and Stanley-Samuelson 1993. Schmidt *et al.* 1978) and one in a marine snail (McIntosh *et al.* 1995). The PLA_2 present in bee venom (group III) is well documented and displays little sequence similarity with snake venoms (groups IA, II A and II B). The sequence of the protein islolated from the marine cone snail venom (group IX) is incomplete, but has been placed in a group of its own because the lack of sequence similarity to any other published PLA_2s. The

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protein associated with the fat body of the tobacco horn worm, which demonstrates PLA_2 activity, has not been isolated in a purified form or sequenced.

1.6 *Limulus* Amebocytes : A Granulocyte Model for Research on the Acute Inflammatory Response

The purpose of the studies described here is to use the *Limulus* amebocyte as a model to study the production of eicosanoids and their role in the acute inflammatory response of an invertebrate blood cell. Five aspects are needed to fully define the eicosanoid biology in a particular system. These five elements include assessing the presence and activity of: 1) fatty acid precursors in the cellular lipids, 2) enzymes capable of releasing these fatty acids from the lipids, 3) enzymes to modify the fatty acids to metabolites, 4) eicosanoid receptor sites and related signal transduction pathways, and finally, 5) eicosanoid degrading enzymes (Stanley-Samuelson and Pedibhotla 1996). It is beyond the scope of this study to completely define all of these aspects.

I have addressed three of these aspects: 1) eicosanoid production by amebocytes, stimulated with calcium ionophore and arachidonic acid, which demonstrates the presence of enzymes to modify free fatty acids; 2) the potential sources of eicosanoid precursors in the phospholipid pools of the amebocyte by determining the fatty acid distribution within the phoshpolipid classes; and 3) studies have been carried out to determine if the amebocyte possesses a means of regulating eicosanoid production. Two potential proteins, in molecular weight (~18 kDa), which reacted in phospholipase A_2 bioassays using sn-2 ^{3}H-arachidonyl labeled *E. coli.* suspension. PLA_2 were detected in the *Limulus* amebocyte. This would be the first reported instance of this type of enzyme isolated from an invertebrate blood cell.

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2.0 Amebocyte Biosythesis of Eicosanoid Metabolites

2.1 Introduction

Invertebrates possess an immune system which mounts a combined humoral and cellular response when challenged by foreign pathogens (Gupta 1991, Stanley-Samuelson *et al.* 1991). Once activated, the cells involved can react through phagocytosis or encapsulation followed by degranulation with the concomitant release of antimicrobial or other cytotoxic materials (Stanley-Samuelson *et al.* 1991). In most arthropods, there are as many as six circulating blood cells: plasmocytes, granulocytes, spherulocytes, oenocytes, adipohemocytes and prohemocytes. Only two of these cells are considered immunocompetent, the plasmocyte and granulocyte (Gupta 1991). In contrast to other arthropods, the primitive marine chelicerate *Limulus polyphemus* has only a single species of circulating blood cells, a granular amebocyte (Armstrong 1979). Therefore, during a cell-mediated immune response, this granulocyte may be capable of carrying out multiple functions. This hemocyte is known to respond during both pathogenic challenge and physical injury through migration of amebocytes to the site, followed by degranulation and the generation of a coagulin clot (Iwanaga *et al.* 1992, Bang 1956, Bursey 1977). The majority of immunological research on *Limulus* amebocytes has focused upon the isolation and identification of compounds stored within the intracellular granules which are involved in the clotting mechanism or have antimicrobial activity (Iwanaga *et al.* 1992). Other than a brief histological survey at the site of injury (Bursey 1977), little work has been carried out concerning the physiological reactions of the amebocyte during an acute inflammatory response.

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In mammals, the major blood cells believed to be primarily involved during the acute initial phase of the inflammatory process are the neutrophil, macrophage and platelet. Gupta (1991) has hypothesized that the *Limulus* amebocyte, as a primitive granulocyte, may function in a manner similar to platelets and macrophages, as well as B- and T-lymphocytes. The acute phase of an inflammatory response is marked by the production and secretion of polyunsaturated fatty acid (PUFA) metabolites, predominately twenty carbon eicosanoids. Presently, there are three primary enzymatic pathways implicated in the production of eicosanoids: cyclooxygenase (CO), lipoxygenase (LO) and cytochrome P-450 monooxygenase (MO). These enzymatic pathways are not all present in a given cell type. For example, in platelets, 12-lipoxygenase and the CO pathways exist; whereas the 5-lipoxygenase and CO pathways predominate in the human macrophage (Gerrard 1985). It can be inferred, therefore, that the differentiation of leukocyte cells has led to selective PUFA oxidation pathways.

Though the most detailed models of cellular response to inflammation have been defined in mammalian blood cells, the last decade has seen an increase in studies of invertebrate tissues and blood cells (Hampson *et al.* 1992, Stanley-Samuelson 1991, DePetrocellis and DiMarzo 1994). The presence of eicosanoids has been reported in over one hundred invertebrates, which implies that there is a fairly high level of conservation for these metabolic pathways (Stanley-Samuelson 1991, DePetrocellis and DiMarzo 1994). In a recent review, De Petrocellis and Di Marzo (1994) emphasized the importance of studying models of eicosanoid biosynthesis in marine organisms. These studies have produced information concerning new enzymatic pathways, revealed the production of unique eicosanoids, and

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demonstrated that eicosanoids have alternative biological activities which in some cases may later be defined in vertebrates. Other authors have postulated that eicosanoid biosynthesis in invertebrates is an essential early step involved in the mediation of the immune response of invertebrates (Stanley-Samuelson *et al.* 1991). Consequently, by studying eicosanoid biosynthesis in a primitive marine species we could gain further understanding about the evolution of the inflammatory response.

This study investigated the production of eicosanoids when *Limulus* amebocytes were stimulated with the calcium ionophore A23187 and the eicosanoid precursor arachidonic acid (AA). Using high pressure liquid chromatography coupled to electrospray ionization mass spectrometry (ESI-MS) several lipid metabolites were qualitatively identified and the major metabolite, 8-hydoxyeicosatetraenoic acid (8-HETE), was quantified. The advent of ESI-MS allows the direct analysis of nonvolatile substances, like fatty acids, without prior derivitization; a step necessary to create volatile compounds for electron impact (EI) and chemical ionization (CI) gas chromatography/mass spectrometry. The quantitative portion of this study demonstrates the ability of the emerging technology of electrospray mass spectrometry to provide a linear response when analyzing nonvolatile small molecules in the picogram to nanogram range.

2.2 Materials and methods

Materials

Arachidonic acid (unlabeled and octadeuterated), the six isomeric forms of hydroxyeicosatetraenoic acid, and Prostaglandin B_2 (PGB_2) standards, were purchased from Cayman Chemical Inc. (Ann Arbor, MI). The 4-bromo-calcium

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ionophore (A23187) and buffer reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Amprep C18 extraction minicolumns were purchased from Amersham International (Arlington Heights, IL). All solvents were obtained from Fisher Scientific (Pittsburgh, PA). Analytical grade absolute ethanol and petroleum ether and HPLC grade water and ethyl acetate were used for the isolation and purification of the eicosanoid metabolites from the C18 minicolumns. All methanol and water used during HPLC separation and ESI-MS analysis were HPLC grade. Prosil (PCR Inc. Gainesville, FL) was used to silanize all glassware. The Zorbax 5μm C18 column (1.0 i.d. x 150 mm) was obtained from Michrom BioResources Inc. (Auburn, CA).

Horseshoe Crabs

The horseshoe crabs, *Limulus polyphemus*, were obtained from either the Marine Biological Laboratory (Woods Hole, MA) or Gulf Specimens (Panacea, FL). They were maintained in fresh, flowing sea water tanks at 12-18°C and fed once a week on a diet of fresh mussels, *Mytilus galloprovincialis*, or frozen sardines. Both male and female horseshoe crabs with a prosomatic carapace width greater than 15 cm were used.

Amebocyte Collection

Prior to blood collection, the animals were rinsed with fresh water and cooled at 4°C for at least one hour to avoid spontaneous degranulation of the blood cells during bleeding. The animals were bled via cardiac puncture at the prosoma-opisthosomic joint using a sterile 16.5 gauge needle. Fifteen milliliter aliquots of

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blood were collected into sterile, siliconized 50 ml Pyrex centrifuge tubes containing 10 ml of ice cold saline (3.0% NaCl, buffered to pH 4.6 with citrate and EDTA) (Armstrong 1985b). The cells were spun in a clinical centrifuge at 300 x *g* for five minutes at room temperature. The plasma/buffer supernatant was decanted and the lightly pelleted cells were resuspended in 10 ml of sterile 3% NaCl. Blood cells were counted using a hemacytometer and average final concentration of cells used for the biosynthesis studies was approximately 2.5×10^7 cells/ml.

Biosynthesis of Eicosanoids.

Using a modification of the method described for mixed crab blood cells (Hampson *et al.* 1992), the amebocytes were stimulated by incubating with (1) arachidonic acid (100 μM final conc.), (2) 4-bromo-calcium ionophore, A23187 (5μM final conc.), or (3) with both arachidonic acid and 4-bromo-calcium ionophore, A23187. This halogenaged derivative of A23187 displays a 10-fold selectivity for Ca^{2+} over Mg^{2+} (Debono *et. al* 1981). In calcium free solutions, A23187 is able to utilize intracellular calcium stores (Schroeder and Strickland 1974, Steinhardt and Epel 1974). Therefore, by increasing the intracellular calcium concentrations, the calcium ionophore promotes cellular activation leading to the fusion of secretory granules with the plasma membrane and degranulation of the cell (Pressman 1976). This promotes the production of eicosanoids by enzymes, phospholipases and lipoxygenases, which are activated during this process. In separate control samples, the cells were incubated with the solvent vehicle. The cells were incubated for 45 minutes with gentle agitation in a 15°C water bath. The reaction was stopped by the addition of absolute ethanol (15% final conc.) to the cell suspension. To assess

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extraction efficiency. 100 ng of PGB_2 was then added to each sample. Quantitative analyses were carried out in triplicate. A parallel experiment was conducted to confirm that metabolite biosynthesis was resulting from the use of exogenous substrate. The cells were treated as above with calcium ionophore (5 µM) and octadeuterated arachidonic acid (100 µM) was substituted as the exogenous substrate.

Extraction Procedures

Using a modification of Powell (1987, 1982), the cell suspension was acidified to pH 3, with 1N HCl, and then centrifuged at 1600 x *g* for 20 min at 4 °C to pellet precipitated proteins. The clear supernatant was loaded on an Amprep C18 minicolumn which had been sequentially precleaned with methanol and water. The polar compounds, salts, neutral lipids and water were then eluted from the column through sequential washes with 15 ml aliquots of 15% EtOH, water, and petroleum ether, respectively. Then the PUFAs were eluted from the column with 10 ml ethyl acetate. The ethyl acetate (EtOAc) fraction was evaporated under nitrogen to a small volume (approx. 100 µl) and stored at -80°C until analyzed.

Liquid Chromatography-Electrospray Ionization Mass Spectrometry (ESI-MS)

At the time of analysis the EtOAc was exchanged for methanol so that the sample solvent would be compatible with the HPLC solvent system used during separation. A 2 µl aliquot of each sample solution was analyzed. The sample constituents were separated on a Michrom UMA HPLC System (Michrom BioResources Inc., Auburn, CA) using a Zorbax C18 column (1.0 i.d. x 150 mm, 5 µm, 80Å) at a flow rate of 50µl/min. The sample was introduced via a 2 µl PEEK

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sample loop into a Valco 10-port electrically actuated injector valve. The qualitative separations were carried out using a linear gradient starting at methanol:water (50:50) and increased to methanol:water (80:20) over 20 minutes, and then run isocratically for an additional 20 min. The ultraviolet absorbance was monitored at 235 nm. Quantitative analyses were carried out by running an isocratic profile of methanol:water (80:20) for 20 minutes, again monitored at 235 nm.

The HPLC effluent was directed into the inlet of an electrospray ionization probe of a Fisons VG Platform II single quadrupole mass spectrometer (Fisons VG Biotech MS, Altrincham, UK). Sample ions were generated in the negative ion mode using nitrogen nebulization assisted electrospray with a source temperature maintained at 70°C. The ESI-MS was tuned daily by direct infusion of arachidonic acid (0.2 mM). The skimmer cone voltage, which controls the degree of fragmentation, was set during tuning for low to moderate fragmentation (-35 to -40V). During qualitative analyses, the mass collection range was 100 to 650 a.m.u. to allow for screening of most eicosanoids. For quantitative analysis the mass collection range was narrowed to 100 to 400 a.m.u., which is more specific for the HETEs. All spectra collected in total scan mode were subjected to background subtraction.

For both qualitative and quantitative analyses, extracted ion profiles were used to locate the molecular ion $[M-H]^-$ for specific metabolites and the PGB_2. Standard curves were constructed from the average area counts of triplicate injections at each concentration level. Area counts were calculated by integration of the extracted molecular ion profile (m/z 319). Standard curves were generated for the six major HETEs because the isomers exhibited different calibration sensitivities ($\partial S / \partial c$). This agrees with previous results seen in thermospray mass spectrometry (Richmond *et al.*

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1986). The PGB_2 was subject to a similar treatment and a standard curve from 0.8 to 12.5 ng was constructed from the molecular ion (m/z 333) to determine levels present in the samples. To evaluate the sensitivity of this technique, separate standard curves were completed for each HETE isomer using both full scan mode and selected ion recording (SIR). The extracted ion profiles from the full scan data were used to generate a curve from the 6.25 to 100 ng level.

2.3 Results

Qualitative Analyses

Qualitative identification of the eicosanoid metabolites present in the samples was based on calculations and a developing ESI-MS library of eicosanoid metabolites (Pace-Asiak 1989, Kerwin and Torvik 1996, Garner *et al.* 1995, Duffin *et al* 1995, Harrison and Murphy 1995a, Wheelan and Murphy 1995). The presence of a number of fatty acids, including eicosanoid precursors, as well as several metabolites were detected in samples. The greatest level of eicosanoids was observed in the amebocytes which had been stimulated with both arachidonic acid and calcium ionophore (Figure 7). Some of the compounds present were found in trace quantities and therefore not readily observed as peaks in the total ion chromatograms. These compounds were detected by extracting their molecular ion chromatogram. Low levels of saturated and monounsaturated fatty acids were detected and they were distinguished mainly by the deprotonated molecular ion $[M-H]^-$, though sometimes the further loss of carbon dioxide [M-45] was observed in the mass spectra of the extracted ion profile. Some of the compounds detected were: myristic acid (m/z 227), palmitic and palmitoleic acids (m/z 255 and 253, respectively), stearic acid (m/z

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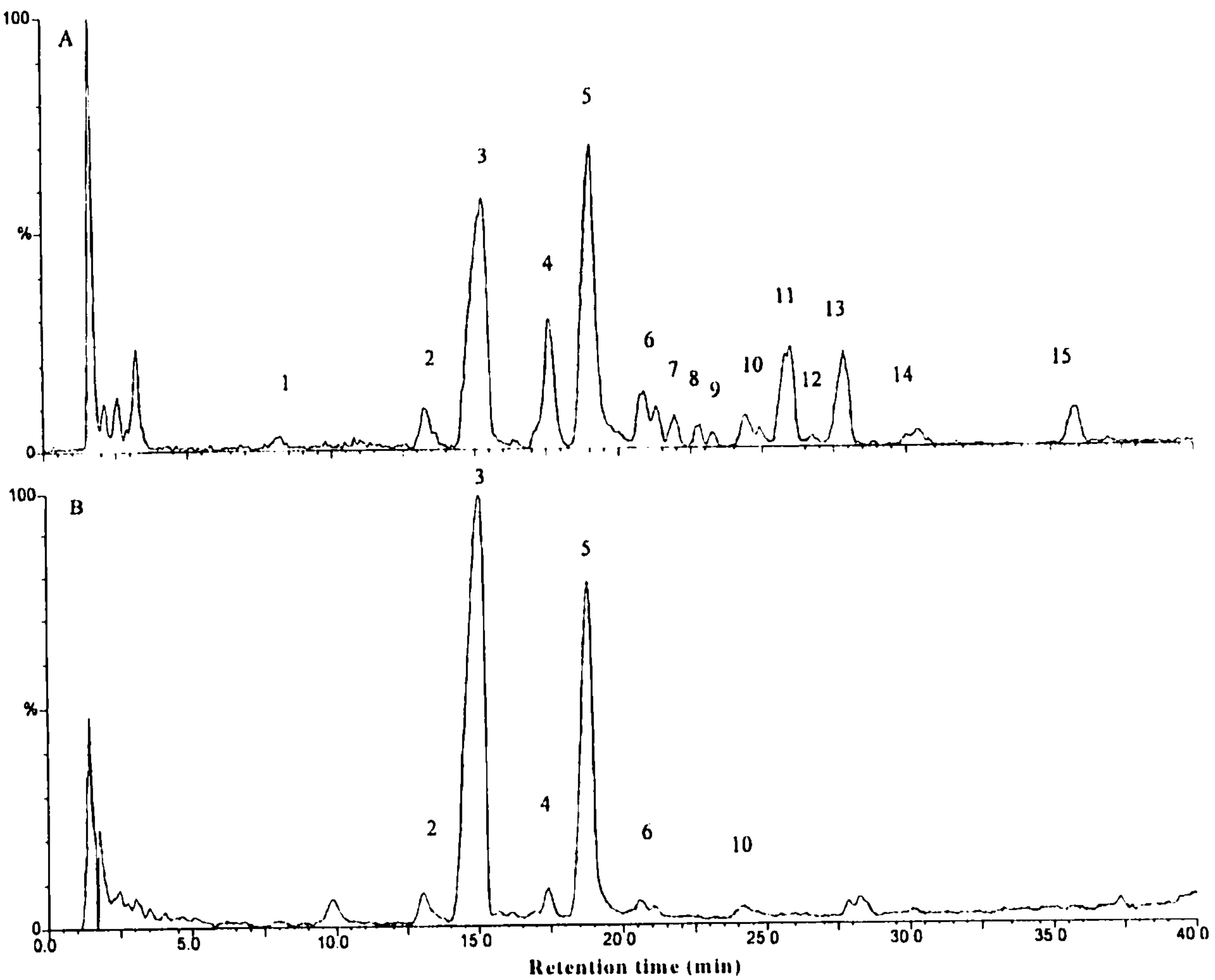

Figure 7. Electrospray and ultraviolet chromatographs of the metabolites by amebocytes stimulated with arachidonic acid and ionophore. The negative ion profile was generated in total scan mode monitoring from 100 to 650 a.m.u. (A) and the ultraviolet spectra was monitored at 235 nm (B).

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283), as well as eicosanoid precursor fatty acids: oleic acid (m/z 281), linoleic acid (m/z 279) and two peaks for linolenic and γ-linolenic acids (m/z 277), eicosatetraenoic acid (m/z 303), eicosapentaenoic acid (m/z 301) and eicosatrienoic acid (m/z 305). In *Limulus* amebocytes, the presence of these fatty acids was also detected during analysis of the total cellular fatty acids (Chapter 3). The major eicosanoid metabolite identified was 8-hydroxyeicosatetraenoic acid (m/z 319). The fragmentation pattern of 8-HETE present in the samples was identical to that seen in the commercial standard (Figure 8). Other minor putative metabolites detected were hydroxyoctadecatrienoic acid (HOTrE) at m/z 293, hydroxyheptadecatrienoic acid (HHTrE) at m/z 279, hydroxyeicosatrienoic acid (HETrE) at m/z 321, hydroperoxy-octadienoic acid (HpODE) at m/z 311, and hydroxyeicosapentaenoic acid (HEPE) at m/z 317 (Figure 9). Since multiple precursor fatty acids were detected in the samples, the appearance of minor metabolites from sources other than arachidonic acid is not unexpected.

Quantitative Analyses

The standard curves for the 5-, 8-, 9-, 11-, 12-, and 15-HETE isomers, generated separately, gave similar response when using either full scan mode (Figure 10) or selected ion recording (Figure 11). The curves demonstrate that the HETEs respond in a linear fashion during ESI-MS analyses to at least the 800 pg level. The signal to background ratio (S/B) for 8-HETE at the 800 pg level was 30:1. This was the lowest level observed for any of the isomers which indicates that the limit of detection for selected ion recording has not been achieved. Therefore, the sensitivity of electrospray ionization displays greater than an order of magnitude improvement

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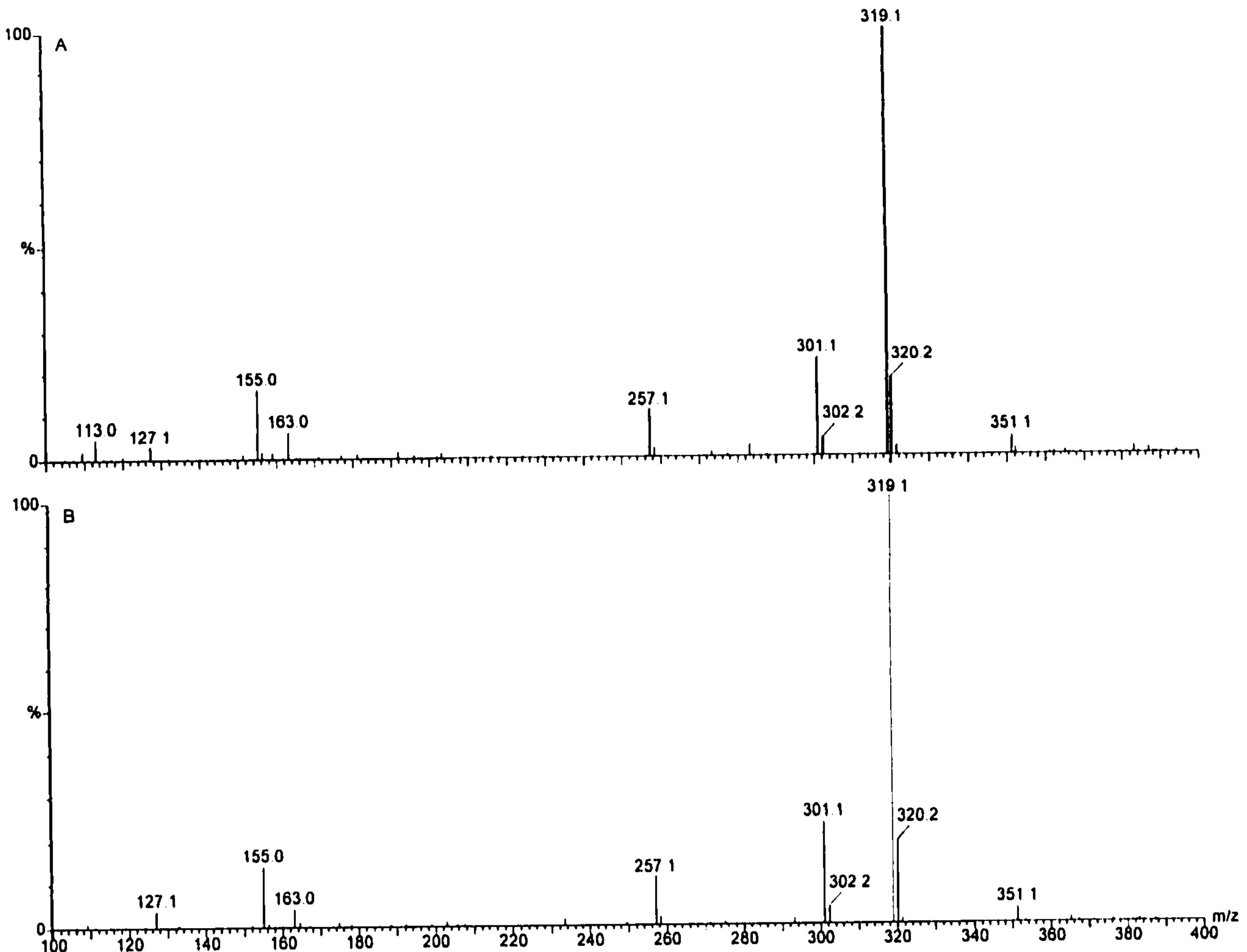

Figure 8. A comparison of ESI-MS mass spectra of 8-HETE generaged from (A) amebocytes treated with arachidonic acid and ionophore, A23187 and (B) a 25 ng commercial standard.

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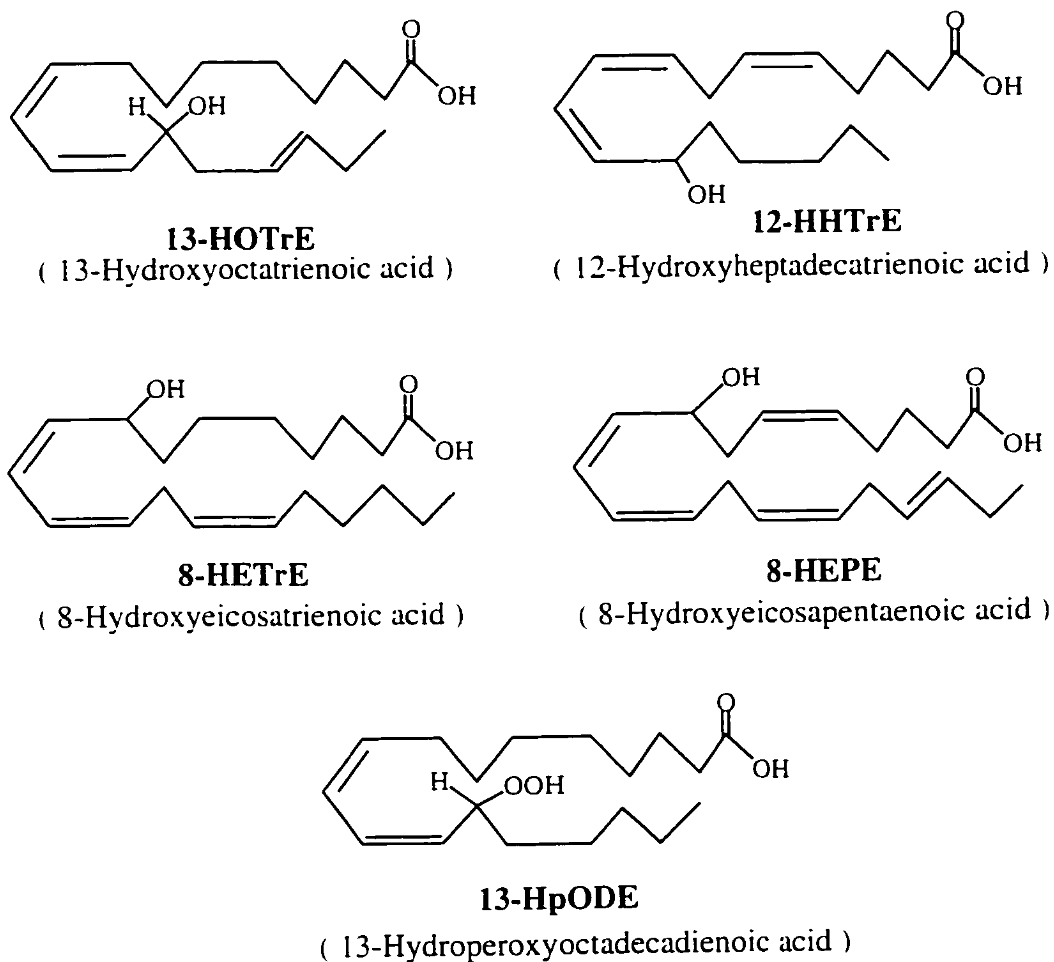

Figure 9. Chemical structures of minor putative eicosanoid metabolites found during the qualitative analysis of samples from amebocytes treated with arachidonic acid and ionophore, A23187.

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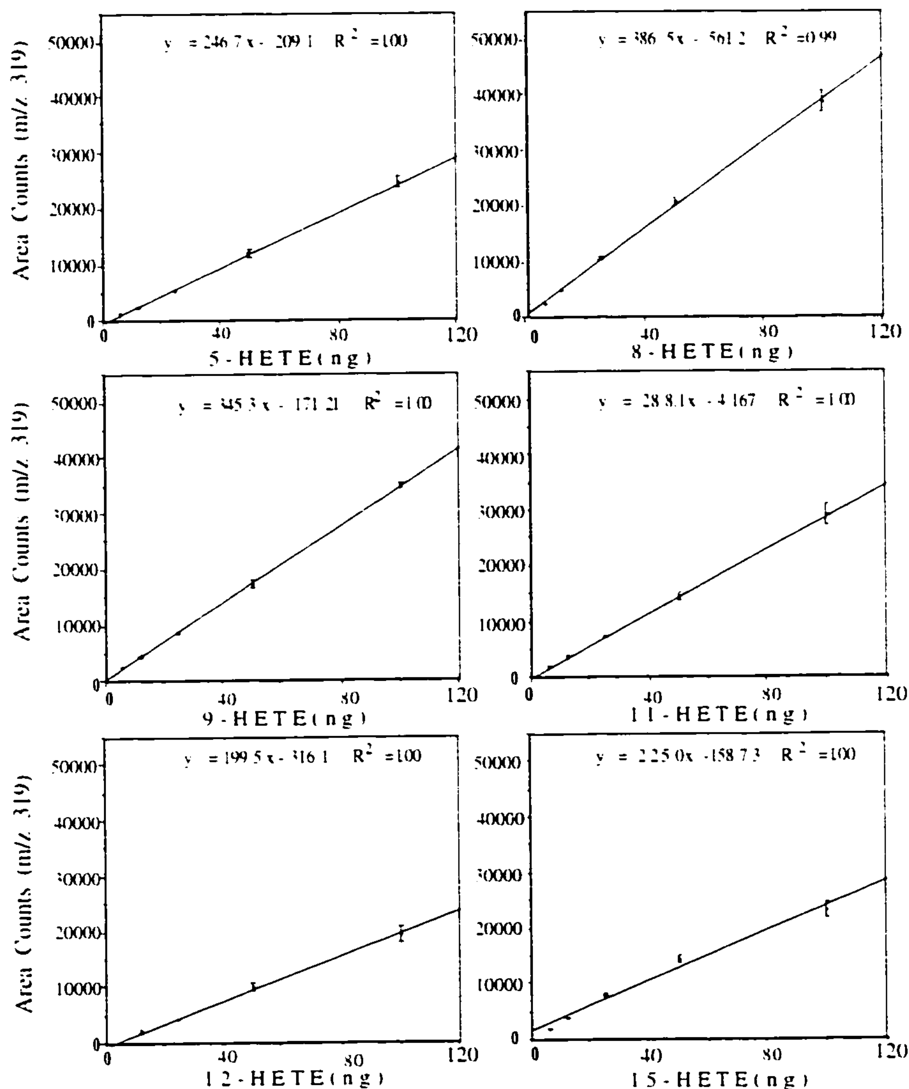

Figure 10. Standard curves for 5-, 8-, 9-, 11-, 12-, and 15-HETE isomers. The HETE standards, 6.25 to 100 ng, were injected and isocratically chromatographed by RP-HPLC. The HPLC eluent was analyzed by ESI-MS in total scan mode of the negative ion from 100 to 400 a.m.u. The area counts were calculated by integrating the area under the curve for the extracted molecular ion profile (m/z 319).

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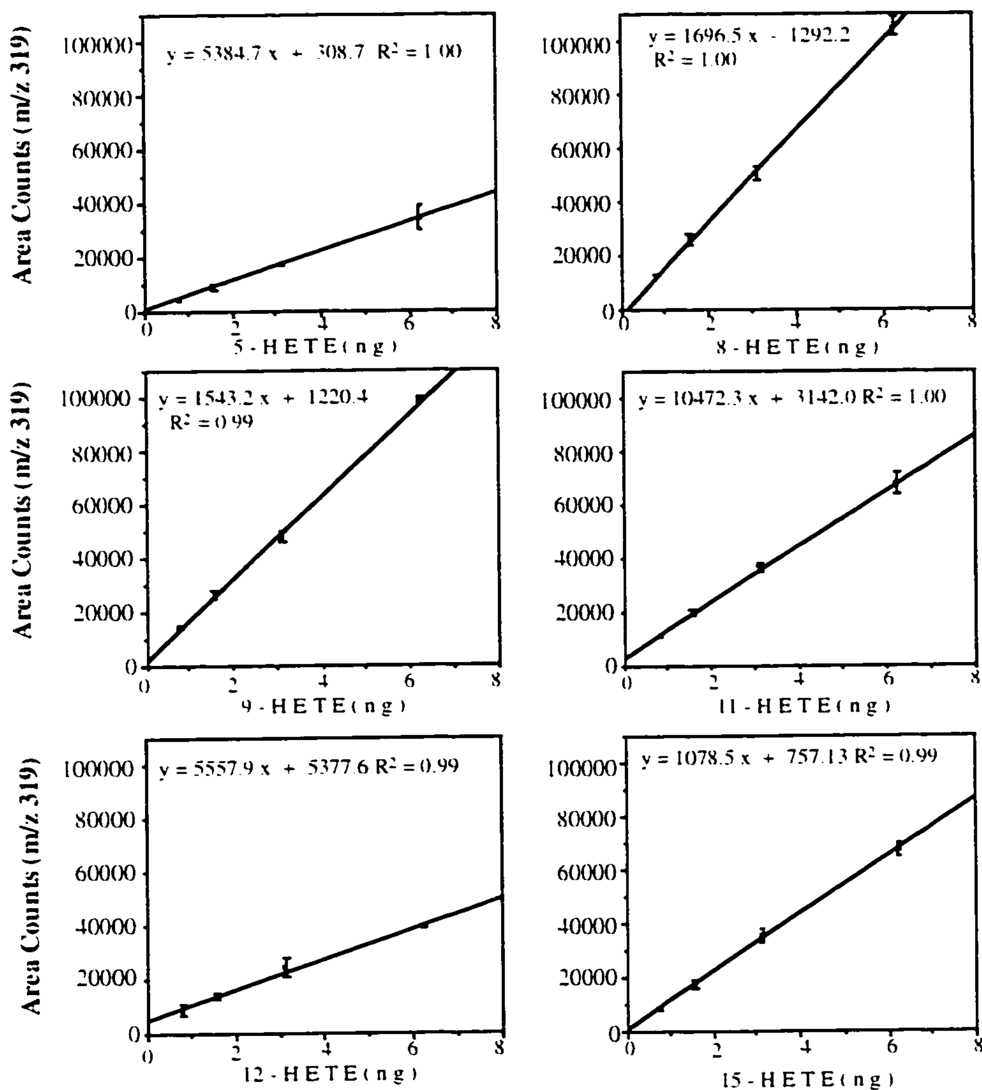

Figure 11. Standard curves for 5-, 8-, 9-, 11-, 12-, 15-HETE isomers. The HETE standards, 0.8 to 6.25 ng, were injected and isocratically chromatographed by RP-HPLC. The HPLC eluent was analyzed by ESI-MS in selected ion recording mode using the fragments listed in Table 1. The area counts were calculated by integrating the area under the curve for the extracted ion profile (m/z 319).

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over the 5 ng limit of detection for HETEs reached using thermospray (Richmond *et al.* 1986). It was possible to identify between five to nine fragments for each HETE isomer. The molecular ion species m/z 319 (M-1, loss of a proton) was present in all isomers, as well as m/z 301 (M-19, loss of water) and m/z 257 (M-63, loss of water and carbon dioxide). All other daughter ions produced by fragmentation of the HETEs were dependent upon the position of hydroxy substitution (Table 1). As it is well recognized that fragmentation in ESI-MS occurs via collision-induced dissociation in the skimmer cone region of the source, it is not unexpected that these fragmentation ions correspond to those observed in FAB MS/MS experiments. In depth discussion of the fragmentation mechanisms can be found in the literature (Wheelan *et al.* 1993, Deterding *et al.* 1992).

Analysis of the quantitative samples indicates that the amebocytes stimulated with exogenous arachidonic acid produced 3.8 ± 0.2 ng 8-HETE /10^6 cells, whereas the hemocytes stimulated with both arachidonic acid and calcium ionophore formed 13.2 ± 2.9 ng 8-HETE /10^6 cells (Figure 12). The level of 8-HETE produced in the controls and cells treated with ionophore alone were at the limit of detection using full scan mode. The percent recovery of PGB_2 for the samples averaged 40%. This level is lower than expected, though the original extraction method (Powell 1982) used Waters Sep-Pak minicolumns which contain four times the packing material of the Amersham minicolumns. Ramis *et al.* (1990) found that leukotriene and HETE recoveries from minicolumns can vary depending upon the packing material and sample treatment after extraction.

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TABLE 1.

Electrospray-mass spectrometry fragmentation patterns produced by the 5-, 8-, 9-,11-, 12-, and 15-HETE isomers.

Compound	Molecular Weight	Base Peak	Fragments
5-HETE	320	319	301, 257, 203, 115
8-HETE	320	319	301, 257, 163,155, 127
9-HETE	320	319	301, 257, 179, 167, 151, 139, 123
11-HETE	320	319	301, 275, 257, 195, 167, 151, 149, 123
12-HETE	320	319	301, 257, 208, 179, 135
15-HETE	320	319	301, 257, 219, 175

The total ion chromatogram of two hundred nanograms of each compound was scanned from 100 to 400 a.m.u. in negative ion mode.

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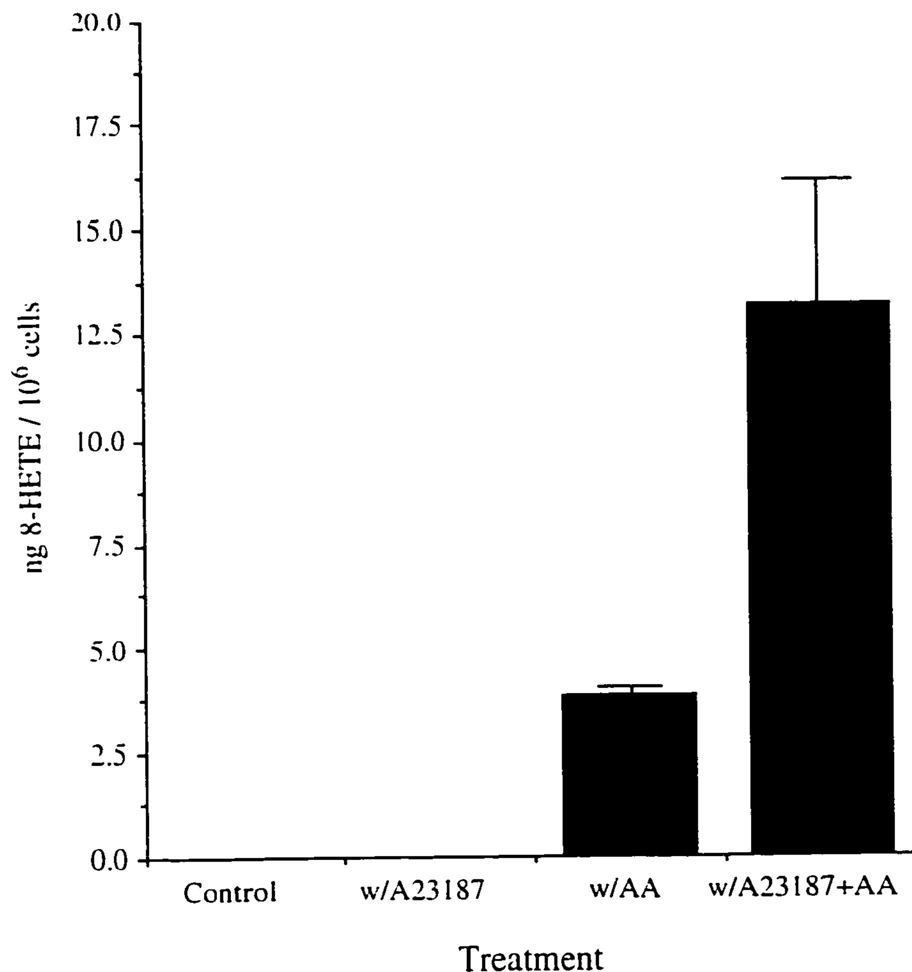

Figure 12. The levels of 8-HETE produced per million cells during four treatments: amebocytes stimulated with vehicle [control], amebocytes stimulated with ionophore, A23187 (5 μM) [w/A23187], amebocytes stimulated with arachidonic acid (100 μM) [w/AA], and amebocytes stimulated with both ionophore and arachidonic acid [w/AA + A23187]. Due to variablity in the PGB_2 recovery, the values have not been corrected for extraction efficiency. Mean ± SD, n = 3-5.

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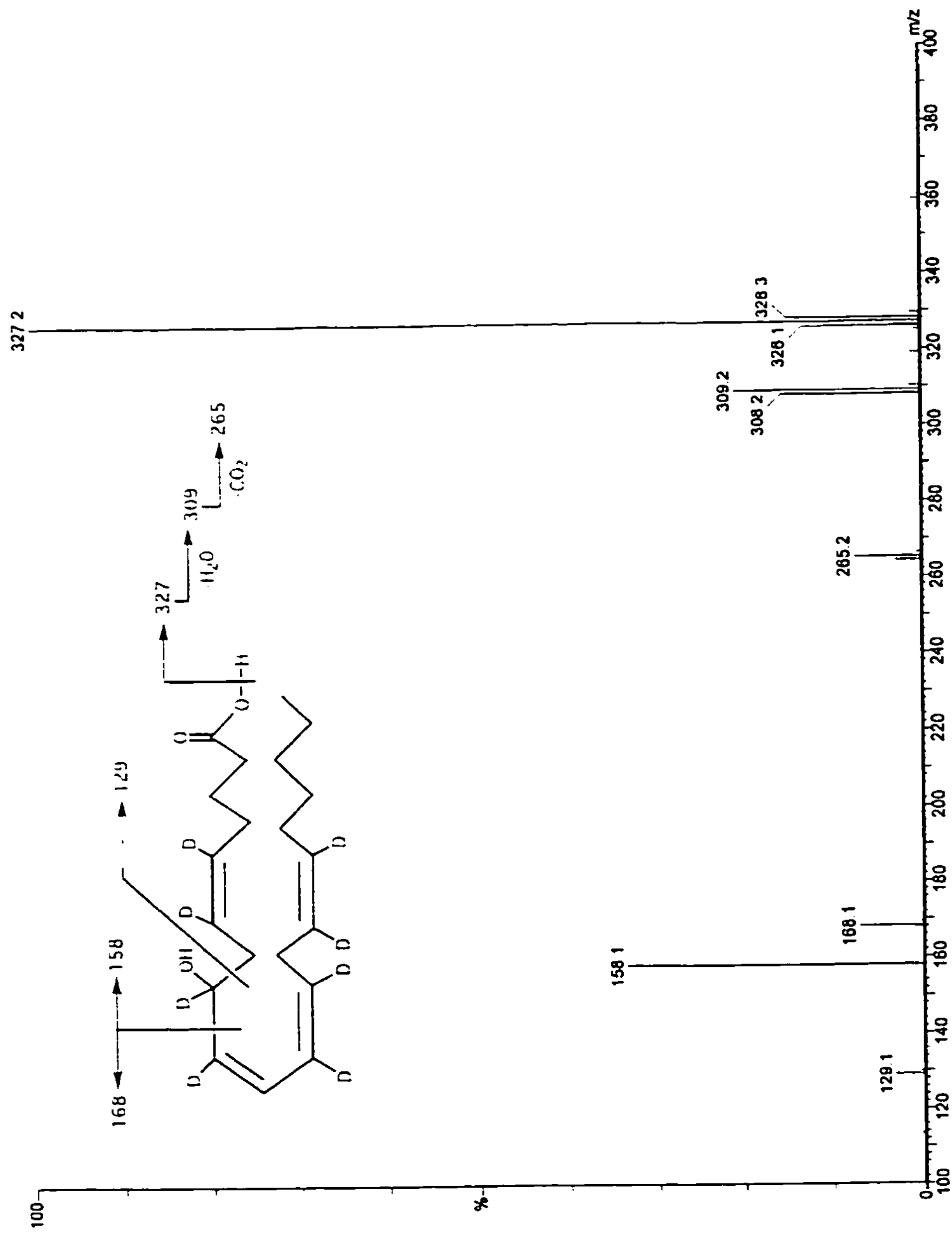

Figure 13. The mass spectra of octadeuterated 8-HETE generated by amebocytes treated with octadeuterated arachidonic acid (100μM) and ionophore, A23187 (5μM).

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Octadeuterated Analysis

The sample incubated with octadeuterated arachidonic acid as the substrate clearly demonstrated the formation of 8-HETE-d_8 (M-H, m/z 327). The mass spectra of the 8-HETE-d_8 peak gave a fragmentation pattern similar to that seen in the samples treated with unlabeled substrate, except for the additional mass from the deuterium. Other than the molecular ion, there were fragments indicating the loss of water (M-19, m/z 309), water plus carbon dioxide (M-63, m/z 265), cleavage prior to the hydroxy group (M-129) and cleavage after the hydroxy group (M-158, M-168) which are 3 to 5 a.m.u. greater, respectively, than their nondeuterated counterpart (Figure 13). These numbers indicate the complete retention of all sites of deuteration throughout the oxidation process which agrees with results seen by Hughes and Brash (1991).

2.4 Discussion

Eicosanoid Biosynthesis

The results indicate that 8-HETE is the major arachidonic acid metabolite produced by stimulated *Limulus* amebocytes. The production of 8-HETE has been observed in both vertebrate and invertebrate organisms. A trend has been noted between these two groups concerning the generation of stereospecific metabolic isomers (DePetrocellis and DiMarzo 1994). In the marine organisms, the R-isomer tends to predominate whereas in vertebrates it is the S-isomer which is the primarily active product. Presently, no enzyme which exclusively produces 8-HETE has been isolated from mammalian cells or tissues (Spector *et al.* 1988), but a stereospecific 8-*R*-lipoxygenase has been detected in homogenates and acetone powders prepared

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from some gorgonians and soft corals (Bundy *et al.* 1986, Corey *et al.* 1987, Brash *et al.* 1987). The production of the stereospecific metabolite, 8-*R*-HETE, has been implicated in the initiation of oocyte maturation in starfish (Meijer *et al.* 1986), and it has also been reported as one of the two major metabolites produced by mixed blood cells in the crab, *Carcinus meanas*, when stimulated with exogenous arachidonic acid (Hampson *et al.* 1992). While the physiological role of 8-HETE in the granular amebocytes of *Limulus* is not known, these primitive immunocompetent cells could be functioning in a manner close to that seen in their mammalian counterparts.

Studies concerning the production of HETEs in mammalian cells have been somewhat skewed toward the 5-, 12- and 15-HETE isomers because they are considered the major metabolites formed. Though the 8-, 9-, and 11-HETE isomers are produced in a variety of cells and tissues, the lack of purified enzymes which specifically lead to the formation of these isomers has hindered their full characterization (Spector *et al.* 1988). Another consideration is that some of these isomers, depending upon cell or tissue type, can be formed by more than one enzymatic pathway. It has been demonstrated that 8-HETE isomer can be produced via both the lipoxygenase and monooxygenase enzymatic systems (Spector *et al.* 1988, Capdevila *et al.* 1986). In general, HETEs are known to influence the inflammatory response. In some tissue injury situations, an increase in HETE formation has been noted and it is felt that these compounds are possibly acting to modulate a number of pathophysiological phenomena (Spector *et al.* 1988). Studies have directly linked 8-HETE production to an acute inflammatory response in mouse skin treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Hughes and Brash 1991, Gschwendt *et al.* 1986). The authors speculated that 8-HETE could play a

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chemotactic role during the host response to immune challenge. In *Limulus polyphemus*, Bursey (1977) noted that within 24 hours of perforation of the exoskeleton that amebocytes had begun to migrate through connective tissue and formed a layer surrounding the wound area. Hemocytes continued to migrate to the site of injury for at least four days. The continued migration of amebocytes to the site of injury indicates the possibility that a chemotactic agent, possibly 8-HETE, is being released.

Electrospray-Ionization Mass Spectrometry

The qualitative electrospray analysis illustrates the power of this method to directly identify eicosanoids formed at low levels from a sample which would normally require much more rigorous sample preparation techniques for structural analysis. Quantitation of eicosanoids, which are secreted at low levels, is nicely demonstrated by the linear response of these compounds using electrospray in both full scan an SIR modes. While common practice dictates the use of an internal standard to correct for the small changes in instrument response, this procedure was not used in the present study as the compound of choice, 8-HETE-d_8, is not commercially available and a suitable alternative was not found. Direct identification and concurrent quantification is preferable for these highly unstable compounds. Although reverse phase HPLC alone can be used to quantify eicosanoids, in many cases this procedure is coupled with either radioimmunoassay to increase sensitivity or derivatization followed by gas chromatography-mass spectrometry (GC-MS) to positively confirm structural identity of the isomers (Young and Girard 1989). Enzyme immunoassays have also been employed for quantitation (Yamamoto *et al.*

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1987). Drawbacks to methods utilizing radioactive isotopes and antibodies include expense and the fact that the availability of these materials is limited to the major eicosanoid metabolites. Therefore these methods, in many cases, are not amenable to reactive intermediates or to uncharacterized metabolites.

For over two decades several forms of mass spectrometry (EI, CI, fast atom bombardment, and thermospray) have served as major tools for identification of lipids and lipid metabolites (Pace-Asciak 1989, Murphy 1993). Gas chromatography-mass spectrometry, using EI and CI, requires derivatization of the metabolites to insure their volatility for analysis (Pace-Asciak 1989, Murphy 1993). Derivatization can interfere with direct quantitation due to (a) the possibility of decomposition during the process and, (b) unwanted side reactions which give nonquantitative yields (Richmond *et al.* 1986, Kim and Sawazaki 1993, Baldwin *et al* 1979). Batch desorption methods, such as fast atom bombardment (FAB) and secondary ion mass spectrometry (SIMS), can analyze nonvolatile compounds directly but are relatively insensitive, difficult to quantify and they are not compatible with on-line detection. The evolution of liquid inlet ionization methods, such as thermospray and ESI-MS, has provided the ability to identify low molecular weight nonvolatile substances on-line without prior derivitization. While the impetus for the development of ESI-MS was for the analysis of large biopolymers, it has been shown an effective technique for the analysis of many smaller nonvolatile molecules (Voyksner 1992). ESI-MS also offers the advantage of detecting compounds in samples irrespective of their UV activity, though normally at least one wavelength is monitored in line to confirm the presence of specific compounds.

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Nonetheless. both qualitative and quantitative analysis of the eicosanoid metabolites were carried out effectively by the use of ESI-MS which demonstrates that this a sensitive and reliable method for the direct identification and quantitation of eicosanoid metabolites. The biosynthesis of 8-HETE as the major metabolite by *Limulus* amebocytes is not inconsistent with eicosanoid formation observed in the blood cells of other marine arthropods. Future studies will serve to clarify the specific physiological role of this eicosanoid and the enzymatic pathways which control the production of this metabolite.

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3.0 Lipid Composition of the Amebocyte

3.1 Introduction

The horseshoe crab, *Limulus polyphemus*, serves as a unique model for studying the lipid chemistry of arthropod blood cells. Due to constraints on organism size and the amount of blood available, the majority of lipid studies previously performed in arthropods have involved analyses of whole animals, muscle tissue or specific organs (Sugiara *et al.* 1992, Chapelle 1987). The presence of as many as six circulating blood cells in arthropods further complicates the isolation and analysis of an individual cell type (Gupta 1991). In contrast, large quantities of blood are available from horseshoe crabs and they possess a single circulating blood cell type, the granular amebocyte (Armstrong 1985a). Moreover, *Limulus* is considered to be a living fossil and its blood cells are regarded as 'primordial immunocytes' as they possess functions comparable to those found in vertebrate leukocytes, such as platelets, macrophages and B- and T-lymphocytes (Gupta 1991). These vertebrate leukocytes are especially active during an immunological challenge from an infection or injury. The activation of these blood cells during an immune response is associated with the production of eicosanoid metabolites. These metabolites are primarily generated from unsaturated twenty carbon polyunsaturated fatty acids (PUFAs) cleaved from the cell's phospholipid membrane, notably di-homo-γ-linoleic (20:3), arachidonic (20:4) and eicosapentaenoic (20:5) acids. The presence of these PUFAs in specific phospholipid pools in the cell membrane has also been implicated in accelerating membrane fusion (Glaser and Gross 1994). The *Limulus* amebocyte has

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demonstrated the ability to produce eicosanoids (MacPherson *et al.* 1996) and its rapid degranulation in the presence of pathogens is also well established (Iwanaga 1993, Armstrong 1985b). 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.

These studies investigate the phospholipid composition of the *Limulus* granular amebocyte. Initially, the cell's fatty acid composition was examined after saponification and methylation of the total lipid. The fatty acid methyl ester profile of the amebocyte lipids was analyzed by gas chromatography-mass spectrometry (GC-MS). In other samples, the total lipid was extracted and fractionated on a silica column to separate the neutral lipid, glycolipid and phospholipid components. The class distribution within the phospholipid fraction was analyzed by high pressure liquid chromatography (HPLC). The major phospholipid classes (PE, PC, PI and PS) were collected individually and subjected to HPLC coupled to electrospray-ionization mass spectrometry (ESI-MS) to determine their molecular species composition. Cardiolipin (CL), sphingomyelin (SPH) and lysophosphatidylcholine (LPC), were screened in a similar manner by ESI-MS to identify major components.

3.2 Materials and Methods

Materials

The chloroform, methanol, water, hexane, isopropanol, and acetone used to extract, fractionate and separate lipids was Fisher Scientific HPLC-grade (Pittsburgh,

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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 and sphingomyelin standards were obtained from Sigma (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL).

Fatty Acid Methyl Ester Analysis

The horseshoe crab blood cells were isolated in the same manner as described in Chapter 2. The amebocyte's total cellular fatty acids were saponified and derivatized directly by a four step process modified from Miller (1985). The blood cells were isolated and washed, as previously detailed, and then transferred to a 10 ml Pyrex test tube with a teflon-lined lid and pelleted in a clinical centrifuge at room temperature (300 x *g*). The supernatant was decanted, the cells lysed and the lipids saponified by adding 2 ml of 3.75 M NaOH/MeOH (50:50, v/v) and heating at 100°C for thirty minutes. Samples were cooled and the free fatty acids were methylated with the addition of 3 ml of 6N HCl/MeOH (65:55, v/v) and heating at 85°C for 10 minutes. The samples were cooled and one ml of Hexane/CH_2Cl_2 (50:50, v/v) was added. The tubes were inverted end-over-end for 3-5 minutes. The aqueous layer was removed and 3 ml of 0.3 M NaOH was added. The tubes were inverted end-over-end for 10 minutes. The upper organic layer was transferred to an amber vial. The samples were placed under nitrogen for either immediate analysis by GC-MS or stored at -35°C until analyzed.

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Gas Chromatography Mass Spectromety Analysis (GC-MS)

FAME samples were analyzed on a Hewlett-Packard 5790 Gas Chromatograph coupled to a VG 70SE double focusing mass spectrometer using a DB-5MS column, 30 m x 0.25 mm i.d. (J & W Scientific, Folsom, CA). A one μl aliquot of the FAME sample was injected. The DB-5 column was held at 50°C for 1 minute, 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 fatty acid methyl esters (PUFA-2) (Matreya Inc., Pleasant Gap, PA) run under the same conditions as the samples. The mass spectra of the dimethyl acetal peaks were compared with known spectra (National Institute of Standards and Technology's [NIST] mass spectroscopy library).

Lipid Extraction and Fractionation

The cells were isolated and washed, as described in Chapter 2, and transferred to a 15 ml, silanized Pyrex test tube with a Teflon-lined cap. The hemocytes were pelleted, the supernatant decanted and then resuspended in 1 ml sterile 3% NaCl. The lipids were extracted from the amebocytes using a modification of Kates (1986). A 3.75 ml aliquot of methanol/chloroform (2:1, v/v) was added to the cell solution. The samples were vortexed, placed on a rotary shaker for two hours and the solution was then centrifuged in a clinical centrifuge for 5 min. The supernatant was transferred to a new tube, the pellet was reextracted in 4.75 ml methanol/chloroform/water (2:1:0.8, v/v/v) and then centrifuged, as before. The supernatants were pooled, a 2.5 ml aliquot of each chloroform and water was added and then the samples were vortexed,

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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, v/v) and stored under nitrogen at -35°C until fractionated.

The samples were evaporated 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 four column volumes (8 ml) of chloroform to elute the neutral lipids, sixteen column volumes (32 ml) of acetone to elute the glycolipids and finally four column volumes (8 ml) of MeOH to elute the phospholipids and sphingomyelins. The fractions were evaporated under nitrogen and stored at -35°C until analyzed.

Straight Phase-HPLC Analysis of Lipid Fractions

The lipid fractions were analyzed using the method developed by Patton *et al.* (1990, 1982). The neutral lipids, glycolipids and phospholipids 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. x 150 mm, 5 µm, 100 Å). The columns were proceeded by an Upchurch precolumn packed with 30-40µm PerisorbA (Oak Harbor, WA). The peak elution was monitored at 205 nm and the separation was isocratic starting with a flow rate of 0.5 ml/min for the first 60 minutes and then increased to 0.8 ml/min for the remainder of the run (approx. 180 min). The solvent phase was water/1M phosphate buffer (pH 7.4)/ isopropanol/hexane/EtOH/ acetic acid (55: 1.2: 485: 376: 100: 0.6, by vol).

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The neutral lipids and glycolipids were analyzed by straight phase-HPLC (SP-HPLC) to determine the relative proportions of these components in the amebocyte total lipid. The peaks resulting from analysis of the phospholipid fraction were collected for further examination by electrospray-ionization mass spectrometry (ESI-MS). Cardiolipin samples were pooled while PC, PE, PS, PI and SPH peaks were collected individually. Phosphate does not easily volatilize and therefore interferes with ESI-MS analysis. To avoid this problem, samples were briefly taken to dryness under N_2 and reextracted to remove phosphate (Kates 1986, Kerwin *et al.* 1994).

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.* (1994). The phospholipid classes were separated on a reverse phase Phenomenex C18 minicolumn (2.0 i.d. x 150 mm, 5µm, 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/min. The samples were separated using a starting solvent phase of 0.5% ammonium hydroxide in water/MeOH/hexane (12:88:0) for three minutes followed by a linear gradient to water/MeOH/hexane (0:18:12) over a 30 minute period. The column was maintained at 20-25°C.

The solvent flow was split post-column (1:3, v/v) and 100 µl of the reverse phase-HPLC (RP-HPLC) effluent was directed into the inlet of an electrospray ionization probe of a Fisons VG Platform II single quadrupole mass spectrometer (Micromass Ltd., Altrincham, UK). The ESI-MS was tuned daily by direct infusion

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of dipalmitoyl phosphatidylcholine (positive-ion mode) or phosphatidylinositol (negative-ion mode) (2 μM). The skimmer cone voltage was set during tuning for moderate fragmentation (-60 to -70V in negative-ion and 40-50V in positive-ion mode). The mass collection range was set between 100 to 1000 a.m.u. for all samples. Sample ions were generated using nitrogen nebulization assisted electrospray with a source temperature maintained at 80°C.

All PI and PS samples were analyzed in negative-ion mode. PE and PC peaks were analyzed in both positive-ion and negative-ion mode. 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. The molecular ion usually represents the entire phospholipid, either $[M+H]^+$ or $[M-H]^-$ depending on the analysis mode, the exception being phosphatidylcholine in negative-ion mode which displays a pseudo-molecular ion of [M-15] corresponding to a loss of a methyl group from the choline head group (Harrison and Murphy 1995b). Since the phospholipids were separated by class prior to ESI-MS, the head group 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 were also used to predict the likely diacyl and alkenylacyl species present in each sample. In the more

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phospholipid classes displaying complicated spectra, phosphatidylcholine and phosphatidylethanolamine, published tables of the three subclasses (diacyl, alkylacyl and alkenylacyl) were used to help determine the molecular species of the minor peaks (Murphy 1993). In these tables, the molecular ion is calculated based 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 (Kerwin *et al.* 1994, Murphy 1993).

Analysis of Lesser Phospholipid Classes

The smaller peaks (< 5%) present in the HPLC phospholipid separation (cardiolipin and sphingomyelin) were screened to identify major species present. The samples were separated by RP-HPLC in a manner similar to the major phospholipid classes. The mass range for analysis was typically set between 100 to 1000 a.m.u., though for cardiolipin samples the mass range was extended to 2000 a.m.u.. These samples were screened in both positive- and negative-ion modes.

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3.3 Results

GC-MS Analysis of Fatty Acid Methyl Esters.

The chromatograph of the fatty acid methyl esters (Figure 14) revealed that almost half (46%) of the amebocyte fatty acid content consisted of two twenty carbon polyunsaturated fatty acids, arachidonic acid (20:4) and eicosapentaenoic acid (20:5) (Table 2). The next most abundant species were the saturated (18:0) and monounsaturated (18:1) eighteen carbon fatty acids at 12.9% and 11.6%, respectively.

Dimethylacetals (DMA) made up nearly twenty percent of the total lipid. Dimethylacetals are formed by the acid methanolysis of phospholipids containing vinyl groups in ether linkages, termed plasmalogens. Therefore, the dimethylacetals here indicate that a considerable portion of the phospholipids contain ether lipids with alkenyl groups at the sn-1 position of the phospholipids[1]. The DMA profile in the amebocyte indicates that eicosene (20:1) was the most common alkyl chain in ether linkage (9.5%), with lesser amounts of octadecane (18:0) and hexadecane (16:0), 5.9% and 1.4%, respectively.

[1] The IUPAC-IUB stereospecific numbering system (sn) for the glycerol backbone of phospholipids is used to assign the positions of substituents as sn-1, sn-2 or sn-3 for the three different carbon atoms in glycerol (Biochemistry J. 1978. **171**: 21-35).

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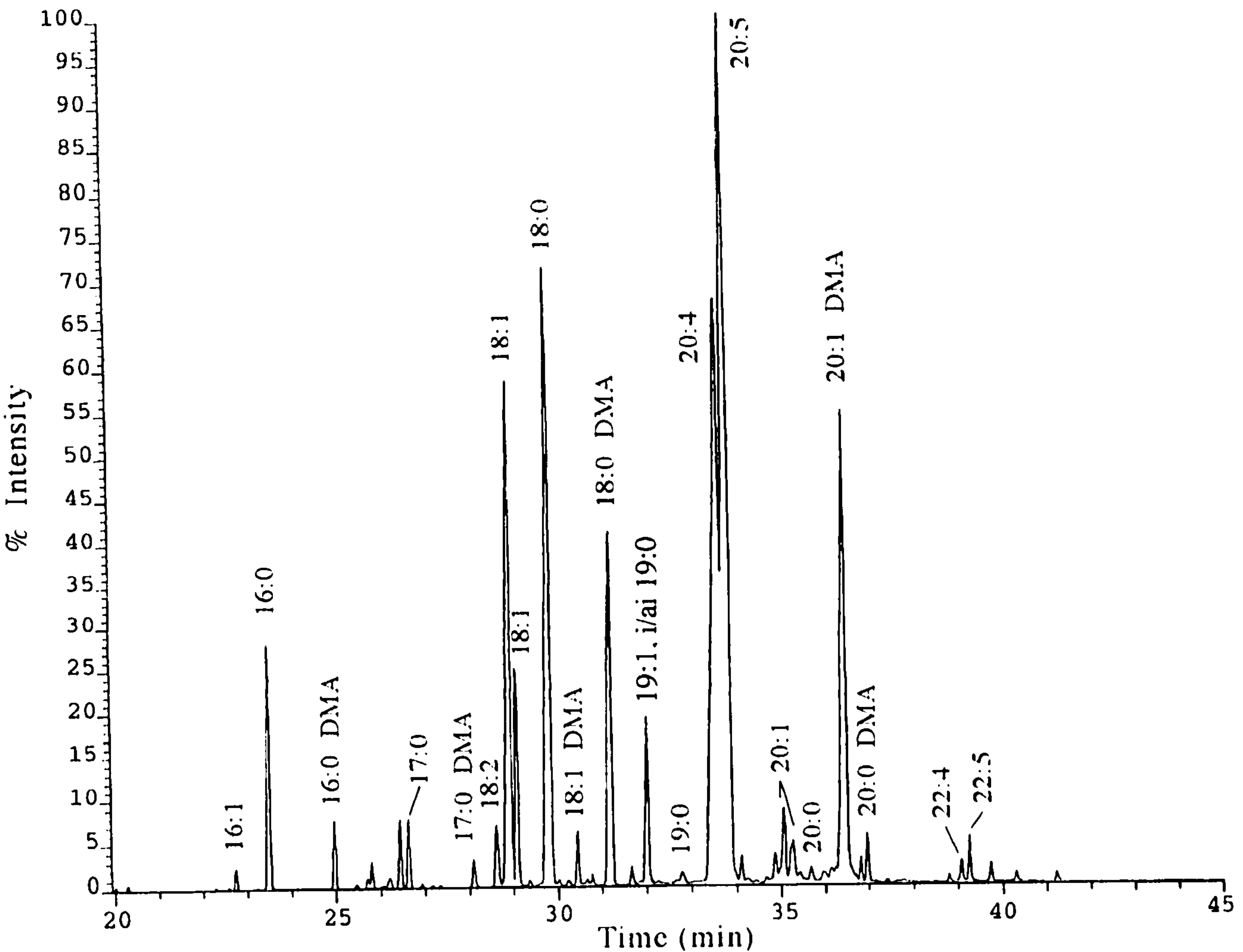

Figure 14. GC-MS chromatograph of the fatty acid methyl esters from the horseshoe crab amebocyte.

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TABLE 2

Amebocyte fatty acid composition determined by GC-MS analysis of the fatty acid methyl esters[a]

Fatty Acid	Percent Area
14:0	0.1 ± 0.1
15:0	trace
16:0	2.2 ± 0.2
16:1	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
18:1, n-9	8.4 ± 0.5
18:1, n-7	3.2 ± 0.2
18:2	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
20:1	0.8 ± 0.3
20:4	25.4 ± 1.0
20:5	20.5 ± 0.8
20:0 DMA	0.5 ± 0.1
20:1 DMA	9.5 ± 1.3
22:0	trace
22:4	0.7 ± 0.1
22:5	0.7 ± 0.1

[a] values are the mean ± SEM, n=5. Trace levels of fatty acid were considered to be less than 0.1%

[b] this peak was a mixture of fatty acids with molecular weights of 294, 310 and 312 a.m.u.

i/ai = iso/anteiso

DMA = dimethyl acetal

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Composition of the Total Lipid

Analysis by straight phase-HPLC revealed that over three quarters of the total cellular lipids were phospholipids (~77%). The remainder of the total lipid was comprised of neutral lipid (~19%) and a small amount of glycolipid (~4%) (Table 3).

Straight-phase HPLC Analysis of the Phospholipid Classses.

There was a clean separation between the major phospholipid classes (Figure 15). Analysis of the phospholipid fraction revealed that there was a greater proportion of phosphatidylethanolamine (42.2%) than phosphatidylcholine (36.2%) (Table 4). Phosphatidylethanolamine and phosphatidylcholine were followed by lower levels of phosphatidylserine and phosphatidylinositol at 9.0% and 6.2%, respectively. Sphingomyelin (4.6%) and cardiolipin (1.6%) were also present in the amebocyte membranes. Lysophosphatidylcholine was detected at trace levels in one of the sphingomyelin samples.

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Table 3.

Composition of the amebocyte total lipid[a]

Neutral Lipid	19.4 ± 4.2
Glycolipid	3.8 ± 1.0
Phospholipid	76.8 ± 4.5

[a] values are the mean ± SEM. n=8

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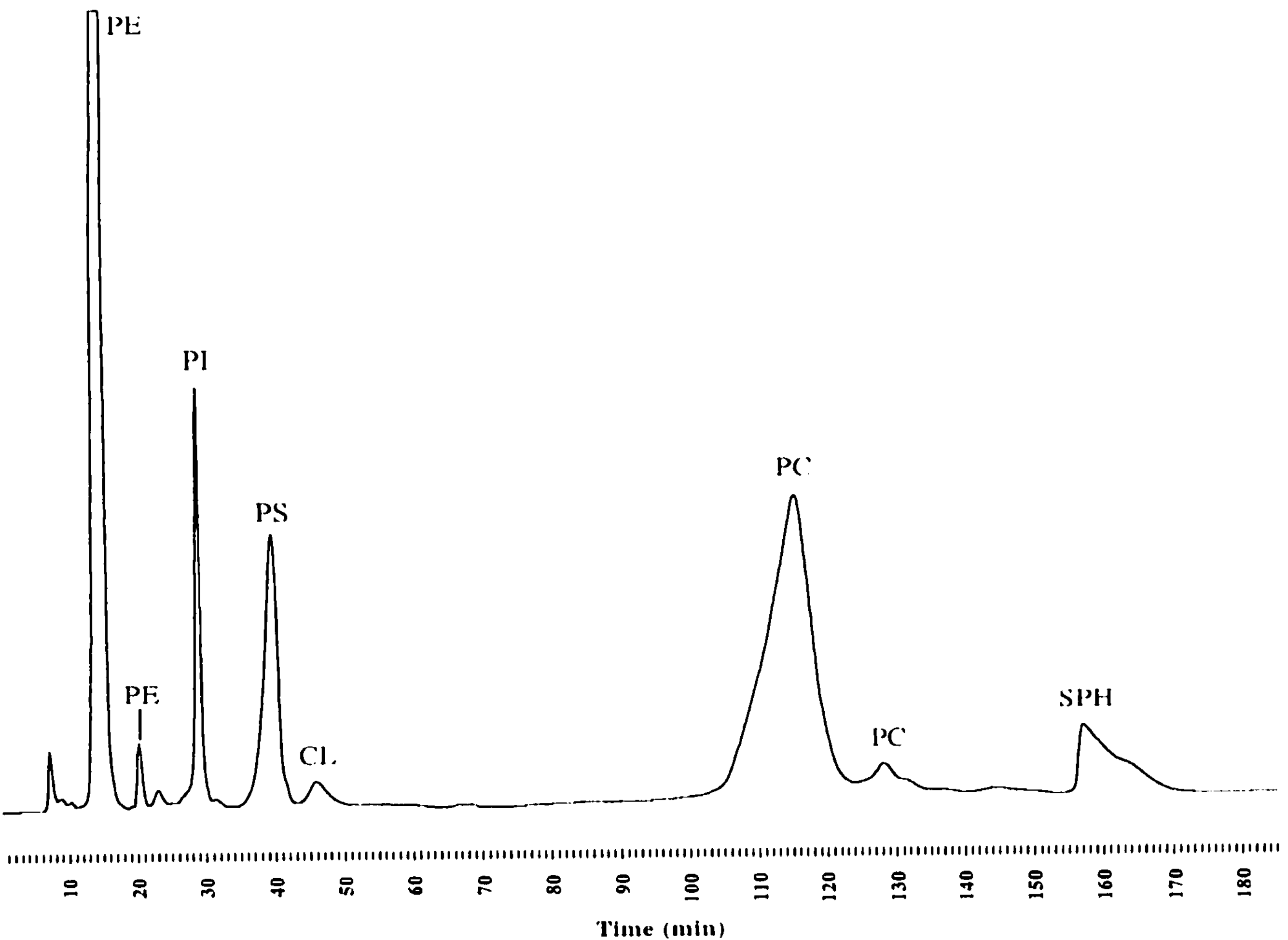

Figure 15. Chromatogram of the straight phase-HPLC separation of the amebocyte phospholipid fraction.

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TABLE 4.

Phospholipid composition of the amebocyte[a]

Phospholipid Class	Percent 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] percent area is the mean ± SEM of amebocyte preparations from seven animals.

[b] one of four samples analyzed by ESI-MS contained small amounts of lysophosphatidylcholine.

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ESI-MS Analyses of the Molecular Species of Each Phospholipid Class.[2]

There are four basic forms of phospholipid (Figure 16). The major group is the diacyl form with fatty acids in ester linkage at the sn-1 and sn-2 postions. Two other forms have alkyl or alk-1-enyl groups in ether linkage at the sn-1 position and a fatty acid in ester linkage at the sn-2 position. The lysophospholipids are formed by the hydrolysis of a fatty acid from one of the prior froms of phospholipid. In diacyl phospholipids. there is a general rule that the sn-1 chain typically tends to be saturated. while the group in the sn-2 position is unsaturated (Kates 1986).

It is not possible to distinguish between alkyl and alkenyl linkages at the sn-1 position using ESI-MS without prior separation of these subclasses (Kerwin *et al.* 1994). Therefore. as previously stated. the phospholipid species were calculated based on the head group and fatty acids fragmentation from the phospholipid molecule. detected in the negative-ion spectra. The calculations of plasmalogen species agreed well with the species of dimethylacetals listed in Table 2. The high levels of arachidonic acid (AA) and eicosapenatenoic acid (EPA) seen in the FAME analyses were reflected in the dominance of phospholipid species containing these fatty acids.

The phosphatidylserine negative-ion mass spectra displayed numerous fragments (Figure 17). 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

---

[2] Phospholipid molecular species nomenclature used here 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=type of linkage at the sn-1 position [a=1-O-acyl. e=1-alkylether. p=1-O-alk-1'-enyl (plasmalogen)]. s=number of carbons at the sn-2 position. t=number of double bonds in the sn-2 chain (Murphy and Harrision 1994).

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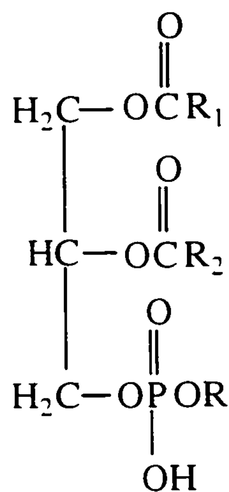
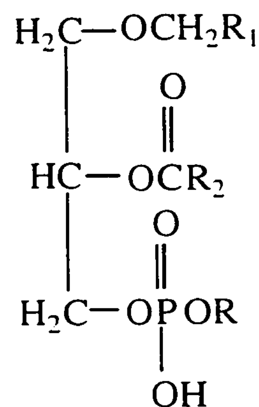
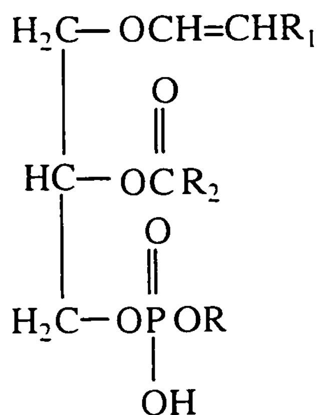


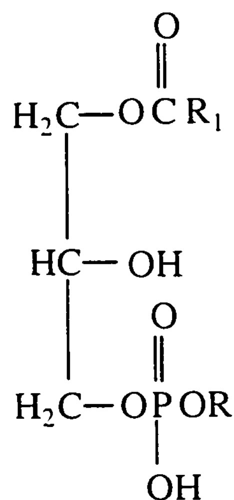
Diacyl Form



Monoalkyl Monoacyl Form



Monoalk-1-enyl Monoacyl Form



Lysophosphatide Form

Figure 16. Common variations of phopholipid structure.

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(m/z 417-439) and free fatty acids (m/z 281, 283). There were two predominant species of phosphatidylserine, 18:0a/20:4 (m/z 810) and 18:0a/18:1 (m/z 788) at 33.8% and 22.7%, respectively (Table 5). Examination of the negative-ion mass spectra for phosphatidylinositol revealed only the molecular ion $[M-H]^-$ (Figure 17). PI was also analyzed at higher skimmer cone voltages (-80 to -100V). These spectra indicated eighteen carbon m/z 281 (18:1) and 283 (18:0), twenty carbon m/z 301 (20:5), 303 (20:4), 305 (20:3), 307 (20:2) and 309 (20:1), as well as 22 carbon m/z 313 (22:6), 315 (22:5), and 317 (22:4) chain fatty acids were present. Greater than half of the phosphatidylinositol (52.3%) consisted of a single species, 18:0a/20:4 (m/z 885). Almost another quarter of the phosphatidylinositol (21.4%) was composed of a mixture of 18:0a/20:5 (m/z 883) and 18:0e/20:4 (m/z 871). The fatty acid composition of PS and PI species (Table 5) indicates that almost ninety percent of these lipids are diacyl species.

Analysis of the positive-ion mass spectra of the phosphatidylethanolamines revealed the molecular ion $[M + H]^+$ and the loss of ethanolamine from the major species [M-142] (Figure 18). The negative-ion mass spectra (Figure 19) gave a molecular ion at $[M-H]^-$, and free fatty acids (m/z 281-303). The PE species consisted of plasmalogen (62%), alkylacyl (11%) and diacyl (27%) phospholipids (Table 6). The chains in the sn-1 position of ether lipids in ether or vinyl ether linkage are not readily cleaved from the phospholipid backbone by ESI-MS analysis. The general lack of saturated and monounsaturated free fatty acids in the negative-ion spectra further supports the predominance of ether lipids in this class. The predominant phosphatidylethanolamine species were 20:1p/20:5 (m/z 776) and 20:1p/20:4 (m/z

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778). There was also a significant amount of 18:0p/20:5 (m/z 750) and 18:0p/20:4 (m/z 752).

The phosphatidylcholine positive-ion mass spectra revealed only the molecular ions [M+H] and the choline head group (m/z 184)(Figure 18). The negative-ion spectra (Figure 19) for the phosphatidylcholines was quite complicated. The pseudo-molecular ion occured at [M-15] which is indicative of demethylation of the choline head group (Harrison and Murphy 1995b). The other peaks present included the losses from the molecular ion of the quaternary nitrogen from the molecular ion (m/z 660-720), the loss of quartenary nitrogen and fatty acids (m/z 370-420), as well as the presence of large levels of free fatty acids (m/z 281-303). Whereas plasmalogen levels dominated in PE, the levels of the three subclasses in PC were almost evenly distributed. The PC species were composed of diacyl (39%), alkylacyl (35%) and plasmalogen (26%) phospholipids. The alkylacyl levels in PC were three times greater than the levels seen in PE. 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 mirror the higher levels of diacyl phospholipid in the PC fraction. There was a broad distribution of molecular species present in PC, but more than a quarter of them were alkylacyl and plasmalogen mixtures of 16:0 with either 20:4 and 20:5 (m/z 764, 766, 768) (Table 7).

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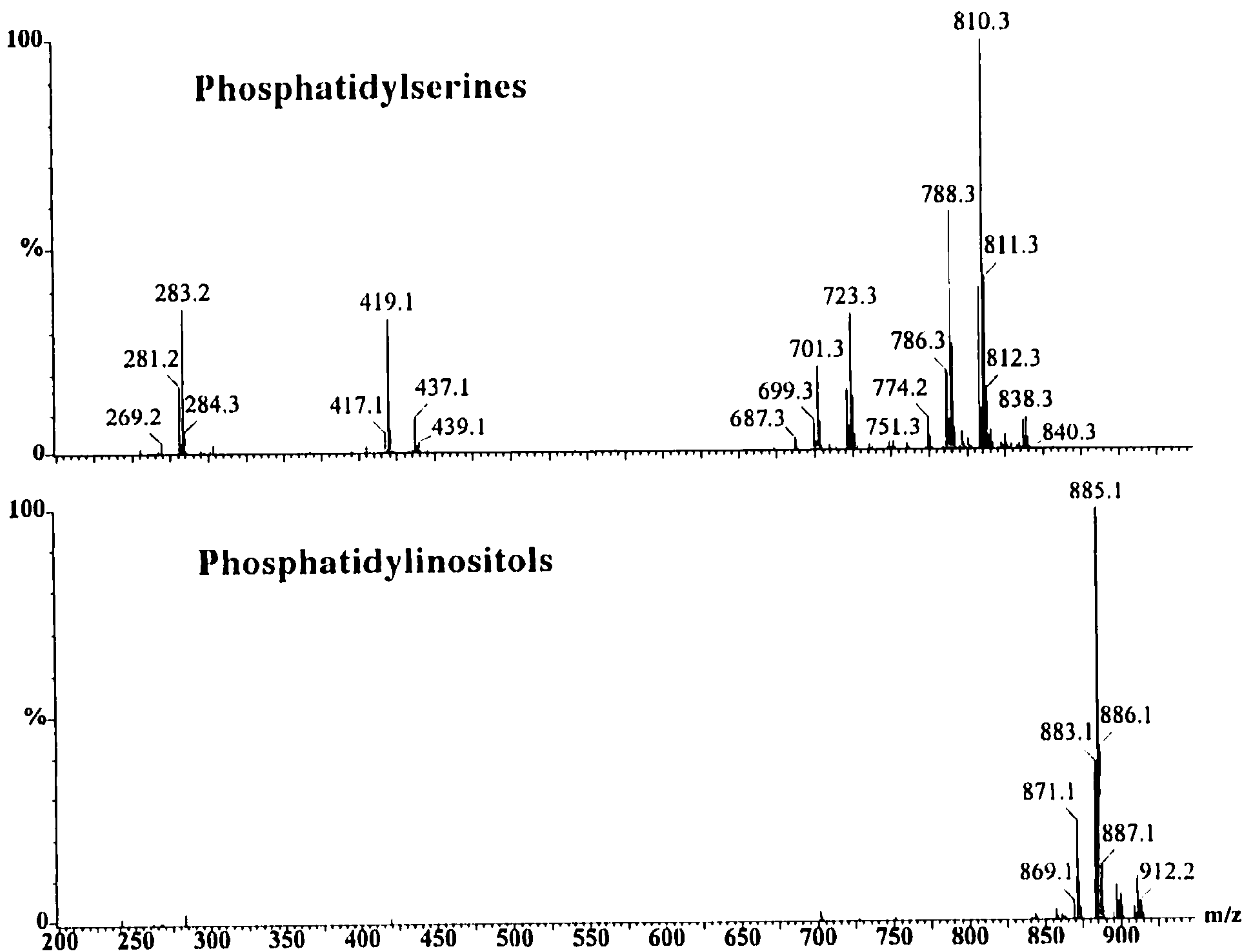

Figure 17. ESI-MS negative-ion mass spectra of the molecular ions and fragments of phosphatidylserines and phosphatidylinositols in the amebocyte.

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TABLE 5

Molecular species composition of the amebocyte phosphatidylserines and phosphatidylinositiols in negative-ion electrospray-ionization mass spectrometry[a,b]

	Molecular species	m/z	% Composition
PS	18:1a/18:1	786.3	4.7 ± 0.6
	18:0a/18:1	788.3	22.7 ± 1.4
	18:0a/18:0	790.3	2.7 ± 0.2
	18:0a/20:5	808.3	3.3 ± 1.2
	18:0a/20:4	810.3	33.8 ± 1.0
	18:0a/20:3	812.3	5.8 ± 0.1
	18:0a/20:2	814.3	4.6 ± 0.6
	18:0a/22:5	836.3	4.3 ± 0.2
	18:0a/22:4	838.3	6.8 ± 0.8
	18:0a/22:3	840.3	1.0 ± 0.2
	18:0e/18:1,18:0p/18:0	774.3	2.8 ± 0.1
	18:0e/20:4	796.3	1.0 ± 0.2
	18:0e/20:3	798.3	0.6 ± 0.2
	18:0p/20:1	800.3	1.3 ± 0.2
	18:0e/20:1	802.3	1.5 ± 0.5
	18:0p/22:4	822.3	0.8 ± 0.1
	18:0e/22:4	824.3	2.2 ± 0.3
PI	16:0a/20:4	857.1	1.1 ± 0.4
	18:0a/18:2	861.1	0.6 ± 0.1
	18:0a/20:5	883.1	14.7 ± 1.3
	18:0a/20:4	885.1	52.3 ± 1.8
	18:0a/20:3	887.1	7.3 ± 0.4
	18:0a/22:6	909.1	1.2 ± 0.1
	18:0a/22:5	911.1	4.5 ± 0.3
	18:0a/22:4	913.1	2.0 ± 0.1
	16:0e/20:4	843.1	0.8 ± 0.1
	18:1p/20:4, 18:0e/20:5	869.1	1.4 ± 0.2
	18:0e/20:4	871.1	6.7 ± 0.6
	18:0e/20:3	873.1	1.0 ± 0.1
	18:0p/22:5	895.1	0.7 ± 0.0
	18:0e/22:5	897.1	3.3 ± 0.3
	18:0e/22:4	899.1	2.4 ± 0.3

[a]phospholipid nomenclature where the letter designates the linkage at the sn-1 position in the phospholipid, where a = 1-O-acyl, e = 1-alkylether and p = 1-alk-1'-enyl (plasmalogen).

[b]the percent composition was based on the average relative intensities of each molecular ion from four independent analyses. Mean ± SEM.

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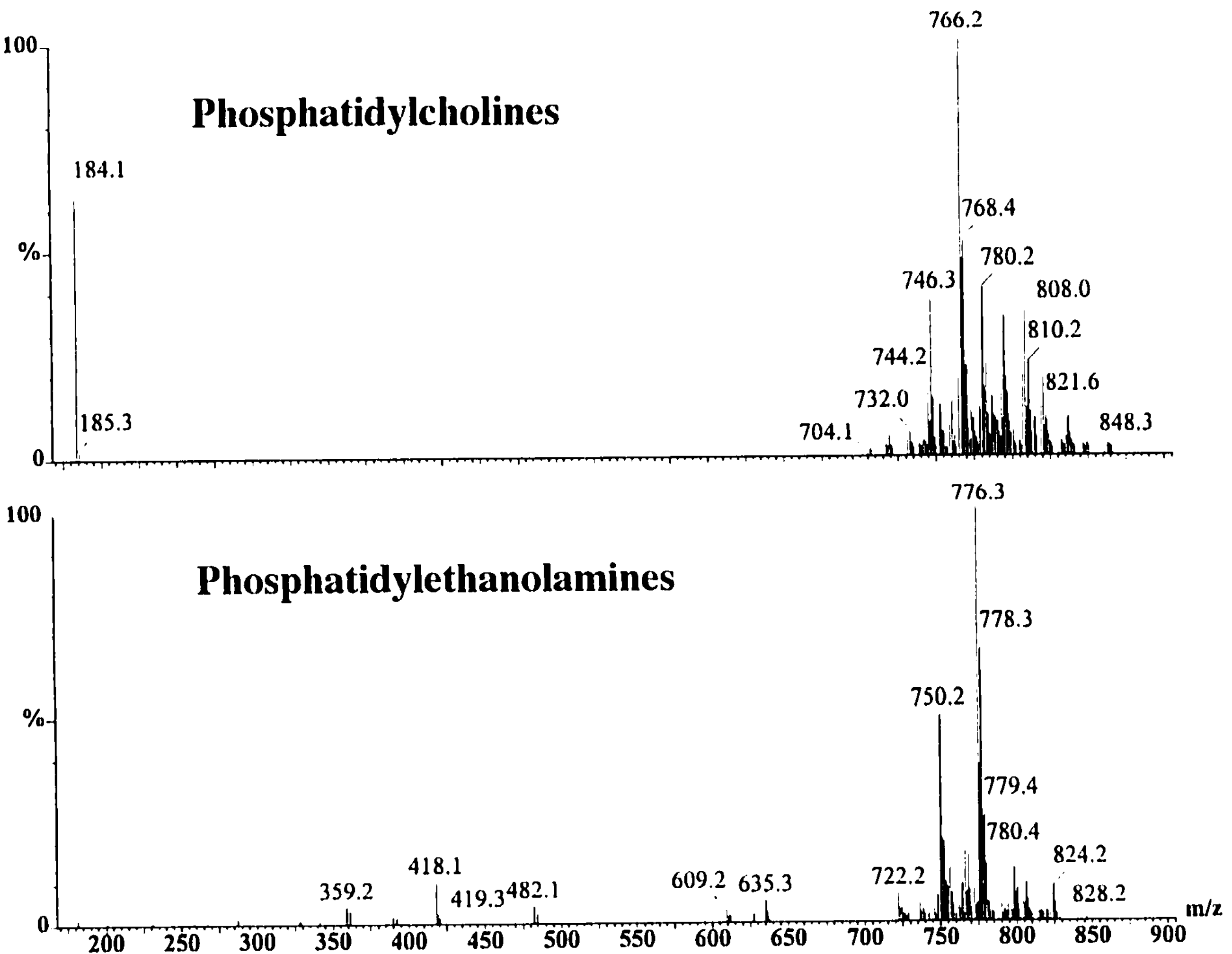

Figure 18. ESI-MS positive-ion mass spectra of the molecular ions and fragments of phosphatidylcholines and phosphatidylethanolamines in the amebocyte.

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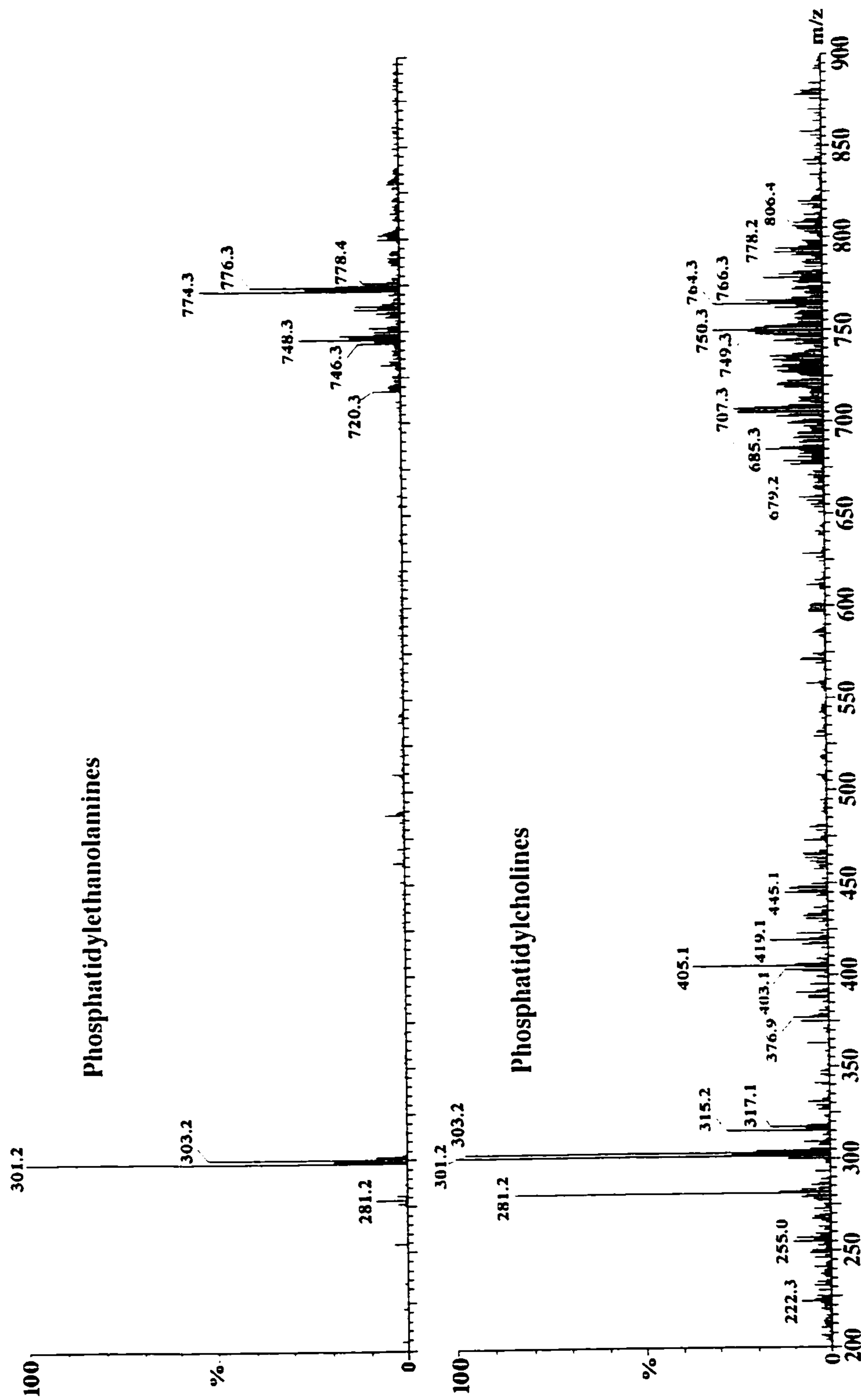

Figure 19. ESI-MS negative-ion mass spectra of the molecular ions and fragments of phosphatidylcholines and phosphatidylethanolamines in the amebocyte.

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TABLE 6

Molecular species composition of the amebocyte phosphatidyl-ethanolamines in positive-ion electrospray-ionization mass spectrometry [a,b]

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

[a]phospholipid nomenclature where the letter designates the linkage at the sn-1 position in the phospholipid, where a = 1-O-acyl, e = 1-alkylether and p = 1-alk-1'-enyl (plasmalogen).

[b]the percent composition was based on the average relative intensities of each molecular ion from five independent analyses. Mean ± SEM.

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TABLE 7

Molecular species composition of the amebocyte phosphatidylcholines in positive-ion electrospray-ionization mass spectrometry[a,b]

Molecular Species	m/z	% Composition	Molecular Species	m/z	% Composition
16:0a/16:1	732.3	1.2 ± 0.2	16:0e/16:0	718.3	0.7 ± 0.1
14:0a/20:5	752.3	2.8 ± 0.5	14:0e/20:5	738.3	1.1 ± 0.2
14:0a/20:4	754.3	1.3 ± 0.2	16:0e/18:2	744.3	2.6 ± 0.1
16:0a/18:2	758.3	1.1 ± 0.0	16:0e/18:1	746.3	6.1 ± 0.5
16:0a/18:1	760.3	2.0 ± 0.1	16:0e/18:0	748.3	1.4 ± 0.3
16:0a/18:0	762.3	0.8 ± 0.1	16:0p/20:5	764.3	4.2 ± 0.4
16:1a/20:5	778.3	1.5 ± 0.1	16:0e/20:5, 16:0p/20:4	766.3	14.4 ± 0.5
16:0a/20:5	780.3	4.8 ± 0.2			
16:0a/20:4	782.3	2.8 ± 0.4	16:0e/20:4	768.3	8.9 ± 1.0
18:1a/18:2	784.3	0.5 ± 0.1	18:1p/18:1	770.3	1.0 ± 0.4
18:1a/18:1	786.3	2.1 ± 0.1	18:0e/18:2, 18:1e/18:1	772.3	1.6 ± 0.2
18:0a/18:1	788.3	2.0 ± 0.2			
18:0a/18:0	790.3	0.9 ± 0.2	18:0e/18:1	774.3	1.5 ± 0.1
18:1a/20:5	806.3	2.5 ± 0.1	18:0p/20:5	792.3	2.7 ± 0.4
18:1a/20:4, 18:0a/20:5	808.3	4.5 ± 0.2	18:0p/20:4	794.3	4.7 ± 0.1
			18:0e/20:4	796.3	2.3 ± 0.1
18:0a/20:4	810.3	3.3 ± 0.3	18:1p/20:1	798.3	0.7 ± 0.1
20:1a/18:2	812.3	1.2 ± 0.1	18:0p/20:1	800.3	1.3 ± 0.1
20:1a/18:1	814.3	1.2 ± 0.1	20:1p/20:5	818.3	1.5 ± 0.3
18:0a/22:6	834.3	1.0 ± 0.1	20:1p/20:4	820.3	2.4 ± 0.2
18:0a/22:5	836.3	1.0 ± 0.1	18:0e/22:5	822.3	1.1 ± 0.1
18:0a/22:4	838.3	0.6 ± 0.0	18:0e/22:4	824.3	0.6 ± 0.0

[a]phospholipid nomenclature where the letter designates the linkage at the sn-1 position in the phospholipid, so a = 1-O-acyl, e = 1-alkylether and p = 1-alk-1'-enyl (plasmalogen).

[b]the percent composition was based on the average relative intensities of each molecular ion from five independent analyses. Mean ± SEM.

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3.4 Discussion

It is difficult to compare the lipid composition of the *Limulus* amebocyte to the blood cells of other arthropods as there currently exists little data for other arthropod species. There is one report of 20% eicosapentaenoic (EPA) and 10% arachidonic acid (AA) levels for the total cellular lipids of the mixed blood cells of the crab *Carcinus meanas* (Hampson *et al.* 1992). The amebocyte can be compared to vertebrate white blood cells with which it shares similar functions; i.e. platelets and macrophages.

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 phosphatidylcholine and phosphatidylethanolamine classes (Paltauf 1994, Akoh and Chapkin 1990). 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 Sugiura *et al.* (1992) the tissues of two arachnids were analyzed, a spider and water scorpion, and they contained between 6.7 to 17.9 % alkylacyl PC and 13.7 to 22.7% alkylacyl PE, respectively. A marine prawn was also reported with 7% alkylacyl PC and 7% alkylacyl PE. It has been reported that alkylacylphospholipids can represent up to 20% of the lipid in marine organisms, and in extreme cases as much as 50-80% (Sugiura *et al* 1992, Chapelle 1987, Malins and Varanasi 1972). The most commonly reported groups in the sn-1 position of ether lipids are 16:0, 16:1, 18:0 and 18:1 (Horrocks 1972), however, the DMA profile in the amebocyte indicates that eicosene (20:1) is the most abundant

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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 it has been previously detected in small amounts at the sn-l position in the ether lipids of human erythrocytes, adrenal glands and placenta (Horrocks 1972).

When considering the distribution of the phospholipid classes, the PE levels in the amebocyte were almost sixty times greater than those seen in platelets and macrophages (23-29%), whereas PC levels were at the lower end of the range normally seen in these leukocytes (35-46%) (Mahadevappa and Holub 1982, Marinetti and Cattieu 1982, Sugiara *et al.* 1982). The amebocyte PI (6.2%) and PS (9.0%) levels were comparable to the amounts of PI and PS previously reported in platelets and macrophages, 6-8% and 5-11%, respectively. The amount of sphingomyelin present in the amebocyte, ~5%, was low in relation to the range of 9-20% sphingomyelin detected in mammalian leukocytes (Marinetti and Cattieu 1982, Sugiara *et al.* 1982). The amebocyte's cardiolipin levels (1.6%) were in a similar range as that reported in human and guinea pig macrophages (1-2.5%) (Marinetti and Cattieu 1982, Sugiara *et al.* 1982).

Within the phospholipid classes, there were a number of overall similarities 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 18:0a/20:4 (41%) and 18:0a/18:1 (37%) (Mahadevappa and Holub 1982), but in the mouse macrophage, PS has three major species 16:0a/18:1 (41.1%), 18:0a/18:1 (19.8%) and 18:0a/20:4 (11.1%) (Akoh and Chapkin 1990). The amebocyte was more similar to the platelet than the macrophage with its

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predominant PS species of 18:0a/20:4 (34%) and 18:0a/18:1 (23%). The PI in the human platelet is almost three-quarter 18:0a/20:4 (71%) while in the mouse macrophage just over half of the PI was 18:1a/20:4 (59.1%). The amebocyte levels of 18:0a/20:4 PI (52.3%) were closer to those seen in the macrophage. The levels of the predominant molecular species in the platelet study may be overestimated as earlier methods used to determine phospholipid species compostion were laborous and typically involved as many as nine steps of separation, derivatization and analysis (Kates 1986). Due to the numerous steps involved, many of the lesser species were likely destroyed or lost during this process.

The major species of phosphatidylethanolamine seen in the amebocyte differed 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 predominant *Limulus* PE species, 20:1p/20:5 and 20:1p/20:4, were unusual with the eicosene (20:1) group in the sn-1 position. There was one prior report, in the guinea pig macrophage, of small amounts of eicosenoic acid, mixed with 18:3, in ester linkage at the sn-1 position, but none in ether linkage (Sugiara *et al.* 1982). The major PE species reported in mammalian leukocytes were 16:0a/20:4, 18:0a/20:4, 16:0a/18:1 and 16:0a/18:2 (Akoh and Chapkin 1990, Mahadevappa and Holub 1982, Sugiara *et al.* 1982). Prior analysis of both *Limulus* nerve tissue and photoreceptor membranes demonstrated high levels of AA and EPA in their phosphatidylethanolamine pools, 45% and 52%, respectively (Benloken *et al.* 1975, Lee and Gonsoulin 1979). In these PE analyses, the levels of EPA were greater than the levels of AA.

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Elevated levels of PE plasmalogen with AA and EPA in the sn-2 position in plasma membranes is quite significant to cellular functions. Glaser and Gross (1994) have shown that the speed of membrane fusion is directly related to the amount of plasmalogen PE molecular species with polyunsaturated fatty acids in the sn-2 position, particularly arachidonic acid. 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 hemocyte degranulation promotes the release of clotting and antimicrobial proteins (polyphemusins and anti-LPS factor) from the cell's granules (Iwanaga 1993). These levels of polyunsaturation may also effect the membrane fluidity of the amebocyte which inhabits a broad range of habitiats, from the continental shelf (~200 m) to shallow bays (~3-5 m) where temperature and salinity ranges are quite extreme (2-3°C/ 32 ppt to +25°C/ 13 ppt) (Shuster 1982).

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 which predominated in mammalian leukocytes were 16:0a/18:1, 16:0a/18:2, 16:0a/16:0 and 16:0e/20:4 (Akoh and Chapkin 1990, Mahadevappa and Holub 1982, Sugiara *et al.* 1982). In *Limulus*, the pattern was similar, except the major species were alkylether and plasmalogens, 16:0e/20:5, 16:0p/20:4, 16:0e/20:4 and 16:0e/18:1. As reported for the PE species, high levels of AA and EPA were also detected in the PC fractions

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of *Limulus* nerve tissue (30%) (Benloken *et al.* 1975) and photoreceptor membranes (42%) (Lee and Gonsoulin 1979).

The presence of 16:0e/20:4 as major species of the amebocyte PC phospholipid pool is significant as it is considered to be a precursor to platelet-activating factor (1-O-alkyl-2-acetyl-PC) (Joly *et al.* 1992). Platelet-activating factor is involved in both signalling and adhesive events between pro-inflammatory cells and vascular tissues (Zimmerman *et al.* 1996). Platelet activating factor-like lipids have been detected in as many as 30 species of invertebrates (Sugiara *et al.* 1992, Chapelle 1987), but this is the first documentation of the presence of a specific precursor to PAF in an invertebrate hemocyte.

In conclusion, the major phospholipid species detected in the *Limulus* amebocyte membranes were 18:0a/20:4 PI, 18:0a/20:4 and 18:0a/18:1 PS, 20:1p/20:5 and 20:1p/20:4 PE and 16:0e/20:5, 16:0p/20:4 and 16:0e/20:4 PC. This phospholipid composition of the *Limulus* amebocyte reflects the key role this hemocyte plays in the immune defenses of this organism. The high levels of polyunsaturated fatty acids in the phospholipids could act as a potential pool for phospholipases for the production of eicosanoids. The 18:0a/20:4 PI species can act as a potential source for the biosynthesis of phosphatidylinositol-4,5-bisphosphate (PIP_2). After cellular activation, PIP_2 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 (Voet and Voet, 1990). The recent detection of a putative IP_3 receptor (Solon *et al.* 1997) in the *Limulus* amebocyte

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reinforces the likelihood that there must be a potential source for IP_3 in the plasma membrane.

The predominance of PE plasmalogen species containing arachidonic and eicosapentaenoic acids could play a significant role in the rapid rate of degranulation of the *Limulus* amebocyte during an immune response to pathogens or external injury. The presence of 16:0e/20:4 PC, a potential precursor to platelet activating factor, in the PC lipid pool is a further indication that signal transduction pathways seen in modern vertebrate cells may also occur in this primitive invertebrate blood cell.

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4.0 Purification and Characterization of a Putative Phospholipase A_2 from the *Limulus* Amebocyte

4.1 Introduction

The *Limulus* amebocyte is an immunocompetent invertebrate hemocyte. Immunological challenge triggers migration of the cells to the site of injury, followed by cellular encapsulation of any foreign bodies and degranulation. Degranulation releases a number of enzymes, coagulation factors and antimicrobial peptides (Iwanaga 1993). Solon *et al.* (1996) have postulated the signal transduction pathway controlling the cell's degranulation is mediated by a G-protein linked receptor. Solon *et al.* (1997) have also identified an IP_3 receptor in the amebocyte which implies the presence of a signal transduction pathway utilizing phospholipase C for the cleavage of IP_3 from PIP_2 from the inner membrane during cellular activation. Inositol trisphosphate binds to its receptor on the endoplasmic reticulum trriggering the release of Ca^{2+}. The Ca^{2+} also acts as an intracellular messenger in a wide variety of cellular responses. An indication of the presence of additional signal transduction pathways in the amebocyte is the production of eicosanoids and a membarane phospholipid composition rich in polyunsatruated fatty acids, especially arachidonic and eicosapentaenoic acids. Eicosanoids themselves can act through autocrine or paracrine signaling and are associated with an inflammatory response.

One of the primary enzymes involved in the initiation of eicosanoid production is phospholipase A_2 (PLA_2). PLA_2s belongs to a signal transduction enzyme superfamily (Dennis 1997). There are two major groups of PLA_2s, the larger cytosolic enzymes (40-85 kDa) and smaller secreted enzymes (13-18 kDa). The

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smaller secreted PLA_2s ($sPLA_2$) have been characterized from the venoms of insects, lizards and snakes, as well as from neutrophils, macrophages and platelets (Mayer and Marshall 1993). As previously described, amebocytes are considered functionally comparable to the mammalian macrophage and platelet (Gupta 1991). The ameobcyte's similarity to mammalian white blood cells and its role in injury response coupled to the production of eicosanoids, makes it a likely candidate to possess a PLA_2. In vertebrates, the induction of PLA_2s and subsequent production of eicosanoids is considered responsible for a number of disease states. The characterization of an $sPLA_2$ from the amebocytes of *Limulus* will contribute to our understanding of the evolution of this biochemical pathway, as well as possible alternate functions in invertebrates.

The putative $sPLA_2$ was purified from Ca^{2+}-ionophore and arachidonic acid stimulated amebocytes using cation-exchange, size-exclusion chromatography and semi-preparative reverse-phase HPLC. During purification, enzymatic activity was assayed using ^{3}H-arachidonyl labeled *E. coli.* suspension. The protein was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and an accurate mass determination was made using HPLC-ESI-MS. The biological activity of the *Limulus* protein and recombinant human PLA_2 ($rhPLA_2$) were compared using a ^{3}H-arachidonic acid *E. coli* suspension. The *Limulus* protein was tested under non-denaturing (boiling) and denaturining (boiling w/ß-ME) conditions, as well as in the presence of phospholipase A_2 inhibitors (manoalide, bromophenacylbromide). The specificity of the lipase activity was investigated by screening the protein in fluorometric triglyceride lipase assay in

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comparison to pancreatic lipase. A partial sequence of the putative enzyme revealed that it has been previously described with an alternative hemagglutination activity (Fujii *et al.* 1992). The hemagglutinin activity of the protein was screened in the presence of human erythrocytes.

4.2 Materials and Methods

Protein Isolation

Amebocyte Crude Extract

The method for purification of the putative PLA_2 was modified from an isolation procedure used to isolate $sPLA_2$ from human rheumatoid synovial fluid (Kramer and Pepinsky, 1991). The isolation and stimulation of the amebocytes was previously described (Chapter 2). The supernatant was discarded and the cells were retained after the stimulation. The cell pellet was resuspended in a minimal volume of extraction buffer (50 mM NaAcetate, 10 mM EDTA, 5 mM PMSF, 1 μM pepstatin, 1 mM iodoacetamide, pH 4.5). The pellet was homogenized with a Tekmar tissue homogenizer (Cincinnati, OH) for 1 minute over ice. The homogenate was then sonicated three times for 30 seconds, frozen to -80°C, thawed and centrifuged at 12,000 x g at 4°C for 20 min. The supernatant was collected, concentrated using an Amicon Diaflo concentrator (Beverly, MA) with a YM-3 filter and protein level was determined by Bradford protein assay (BioRad, Hercules, CA).

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Cation-Exchange Column

The crude protein concentrate was fractionated on a SP-Sepharose Fast Flow (Pharmacia, Sweden) cation-exchange column (49 x 1.6 cm, 90 ml/hr) preequilibrated in 200 mM NaCl, 50 mM NaAcetate, pH 4.5. The column was run with a step gradient from 200 mM to 2M NaCl in 50 mM NaAcetate, pH 4.5. Five salt concentrations in 50 mM NaAcetate, pH 4.5 were used (200 mM NaCl, 600 mM NaCl, 1.0 M NaCl, 1.4 M NaCl and 2.0 M NaCl), 6 ml fractions were collected. The protein elution was monitored by testing each fraction using the Bradford protein assay. Every third fraction was screened for PLA_2 activity using sn-2 ^3H-arachidonate labeled *E. coli* suspension for activity. Peaks displaying PLA_2 activity were pooled and concentrated by Amicon Diaflo concentrator (YM-3 filter) and the percent recovery was determined by the Bradford protein assay.

Size-Exclusion Column

The Sepharose concentrate was separated by gel filtration using a Sephadex G-50 superfine column (50 x 1.6 cm, 2 ml/hr). The column was preequilibrated with 300 mM NaCl, 50 mM NaAcetate, pH 4.5. The column was run isocratically in the same buffer and 1.0 ml aliquots were collected. Protein levels were determined using the Bradford protein assay. Every third fraction before protein elution, every fraction during protein elution and every third fraction after protein elution was tested for activity with the *E. coli* suspension. Active fractions were pooled and concentrated by Amicon Diaflow concentrator with a YM-3 filter. The amount of protein recovered was determined using the Bradford protein assay.

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Reversed-Phase High Pressure Liquid Chromatography

The Sephadex concentrate was separated using a semi-preparative Whatman PRP-3 C18 column (7.0 mm i.d. x 305 mm, 3 ml/min, λ = 280nm, AUFS = 0.02). A maximum of one milligram of protein was injected during each HPLC run to avoid overloading the column. The proteins were separated using a gradient of Buffer A: acetonitrile w/0.1% trifluoroacetic acid (TFA) and Buffer B: H_2O w/0.1% TFA. The gradient profile was A:B (0:100) to (75:25) over a 45 minute period. 1.5 ml fractions were collected. Ten fractions prior to protein elution, all fractions during protein elution and ten fractions after protein elution were assayed for PLA_2 activity with *E. coli* suspension. The acetonitrile was allowed to evaporate off the fractions for 24 hours at 4°C before concentration using Amicon Micron-3 concentrators. Protein recovery was determined using the BioRad Bradford protein assay.

Electrospray-Ionization Mass Spectrometry Analysis

The RP-HPLC concentrate was analyzed twice by electrospray-ionization mass spectrometry (ESI-MS) for molecular weight estimation. A 10 ul aliquot of the concentrate (9.1 μg/ml) was separated on a Zorbax C18 column (150 x 1 mm, 50 μl/min, 300Å column, λ = 214 nm) using a Michrom BioResources UMA HPLC System (Auburn, CA) with a gradient profile with Buffer A: 2% acetonitrile/98% H_2O and Buffer B: 90% acetonitrile/10%H_20, both w/0.1% TFA. The profile was A:B (95:5) to A:B (5:95) over 1 hour. The proteins were analyzed in postitive-ion mode.

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The HPLC effluent was directed into the inlet of an electrospray-ionization probe of a Fisons VG Platform II single quadrupole mass spectrometer (Micromass Ltd.. Altrincham. UK). The ESI-MS was tuned by direct infusion of horse myoglobin (50 pM). The skimmer cone voltage was set at 40V. The mass collection range was set between 0-2400 atomic mass units. Sample ions were generated using nitrogen nebulization assisted electrospray with a source temperature maintained at 90°C. During the first analysis the molecular weights were estimated in total scan mode using centroided data. For an accurate mass determination. the second analysis was collected in continuum mode and subjected to background subtraction prior to deconvolution of the protein envelope using MaxEntropy software (Micromass Ltd. Altrincham. UK).

BioRad Protein Assay

In a 96-well microtitre plate. 10 µl aliquots of column fractions or concentrates were analyzed using the BioRad Bradford protein assay. Protein estimates were made by concurrently running a standard curve (100 - 1200 µg/ml) using BioRad bovine plasma gamma globulin standard (1.53 mg/ml). A 250 µl aliquot of dye solution (1:4. BioRad Bradford dye concentrate:water) was added to each well. After 5 minutes. the plate was read on a microplate reader at 590nm (Molecular Devices. Sunnyvale. CA).

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Protein Concentration

Depending on the volume, protein fractions from the columns were either concentrated using a 50 ml Amicon Diaflo Ultrafilter with a YM-3 filter (3 kDa MW cutoff) or with 1.5 ml Amicon Micron-3 microconcentrator tubes (3 kDa MW cuttoff) (Beverly, MA). For the Diaflo Ultrafilter, molecular weight cutoff filters were prewetted for one hour in distilled water to remove glycerol and fixatives prior to use. The proteins were concentrated at 4°C under nitrogen at 70 psi. For the Micron-3 tubes, the samples were centrifuged in an Eppendorf Centrifuge 545C at 11,000 rpm at 4°C.

Escherichia coli Phospholipase A_2 Bioassay

All PLA_2 enzymatic activity was measured using sn-2 ^{3}H-arachidonyl labeled *E. coli* suspension (Dupont NEN, Wilmington, DE). The method is a modification of Bolognese and Marshall (1993). The commercial suspension consists of lysed *E. coli* cell membranes enriched with ^{3}H-arachidonic acid at the sn-2 position of cardiolipin (44.8%), PE and PG (36.5%) and the remaining in the PC, PS and PI. The PLA_2 cleaves the radiolabeled fatty acid principly from the PE and PG phospholipid pool pools (Loeser *et al.* 1990).

The *E.coli* suspension was defrosted, washed twice in buffer (150 mM NaCl, 25 mM HEPES, pH 7.4) and resuspended in reaction buffer (150 mM NaCl, 25 mM HEPES, 12.5 mM $CaCl_2$, pH 7.4). A 10µl aliquot of *E. coli* suspension, 10µl of enzyme, and 30µl of reaction buffer were combined in a 1.5 ml Eppendorf centrifuge tube. When proteins isolated by RP-HPLC were assayed, BSA (1 mg/ml) was added

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to the procedure (sample:BSA soln., 1:1). In addition to the *Limulus* protein samples, duplicate negative controls (10 µl reaction buffer) and positive controls (10 ng recombinant human $sPLA_2$) were run. The recombinant human 14 kDa PLA_2 ($rhPLA_2$) was kindly provided by Dr. Lisa Marshall (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). Samples were incubated for 30 min at 37°C and the reaction was stopped by the addition of 1 ml ice cold tetrahydrofuran (THF). Each sample was then spiked with 10µl aliquot of ^{14}C-arachidonic acid to determining percent recovery from the minicolumns.

Each sample was loaded onto a 1 ml Waters Sep-Pak amino propyl mini-column (Milford, MA) prewetted with 1 ml THF:H_2O (20:1). The samples were eluted across the columns by gravity flow, this solution was discarded. The free fatty acids were eluted from the column with 1 ml THF:acetic acid (49:1). A 300 ul aliquot of each sample was transferred to a 4 ml plastic scintillation vial containing 3 ml Fisher ScintVerse E (Pittsburgh, PA) and counted on a LKB Wallac 1219 Rackbeta scintillation counter for 5 min. To calculate total suspension counts and total spike counts, duplicate 10 µl aliquots of the *E. coli* suspension and the ^{14}C-arachidonic acid were also counted each time the assay was performed.

Protein Characterization

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The concentrates from each purification step were analyzed by SDS-PAGE. A 10 µl aliquot of protein was added to 40 µl sample buffer [DI water (3.8 ml), 0.5M Tris, pH 6.8 (1.0 ml), glycerol (0.8 ml), 10% SDS (1.6 ml), ß-mercaptoethanol (0.4

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ml), 0.5% bromophenol blue (0.4 ml)]. The samples were heated to 95°C for 5 minutes and 5-10 µl aliquots (5-10 µg protein) were loaded into the wells of a precast BioRad 15% Tris-Glycine gel. The gels were run using a BioRad Mini-PROTEAN II Electrophoresis chamber. The proteins were run into the 4% stacking gel for 15 min at 70 volts, the voltage was then increased to 110 volts for the remainder of the run (approx. 2 hrs.). The *Limulus* proteins were compared to lanes containing recombinant human 14-kDa $sPLA_2$ and a low molecular weight protein ladder (14.5, 21.5, 31, 45, 66 and 97.4 kDa). The proteins were visualized using Coomassie Dye (1.25 g Coomassie Brilliant R-250 in isopropanol:acetic acid:water, 125:50:325, v/v/v) for 30 min and destained in a dilute acid solution (acetic acid:water, 70:830, v/v) overnight.

Biotinylated Western Blot Analysis

The *Limulus* protein concentrate from the RP-HPLC and $rhPLA_2$ samples were separated by SDS-PAGE, as just described, though levels of total protein loaded per well was lowered to below 1 µg. Two negative controls were also run. The first was lysozyme, a 14.5 kDa protein similar in molecular weight to $rhPLA_2$, and the other was limulin, a 55 kDa lectin isolated from *Limulus* hemolymph. The biotinylated Western blot analysis is more sensitive than regular blots (detection limit 5 pg) and total protein levels exceeding 1 µg result in non-specific 1° antibody binding. The proteins were transferred from the gel (100V for 1 hour) on to Millipore Immobilon 0.45µm nitrocellulose (Bedford, MA) using a BioRad Mini Trans-Blot apparatus.

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The nitrocellulose was rinsed in 0.05% Tween-Tris buffered saline (TTBS) for 30 minutes. The blot was then placed in blocking solution (5% nonfat milk in TTBS) for 1 hour at room temperature (RT) on a rotary shaker. The blot was then washed in TTBS, 2 x 10 min at RT, and incubated in the polyclonal, rabbit anti-human recombinant 14-kDa PLA_2 primary antibody (1:6000) (Cayman Chemical, MI) overnight at 4°C on a rotary shaker. The blot was then washed, 2 x 10 min in TTBS at RT, and placed in 2° antibody (biotinylated goat anti-rabbit antibody) (1:6000) at RT for two hours on a rotary shaker. The blot was washed, 2 x 10 min in TTBS, and then placed in a conjugated streptavidin-biotinylated alkaline phosphatase solution for 2 hrs at RT on a rotary shaker. The blot was washed, 4 x 10 min at RT in TTBS on a rotary shaker. The bands were visualized using BioRad 5-bromo, -4-chloro, -3-indoyl phosphate / nitroblue tetrazolium (BCIP/NBT) solutions. After resolution of bands the blot was washed in DI and dried between filter paper.

Protein Sequencing

The proteins from the RP-HPLC were separated by SDS-PAGE, as described above. The proteins were then electrophoretically transferred, as described above, except the nitrocellulose was replaced by a 0.2 μM polyvinylidene difluroide (PVDF) membrane. After blotting, the PVDF membrane was rinsed in DI water (3 x 5 min), stained in Coomassie dye (0.025% Coomassie Brillant Blue R-250 in 40% MeOH) for 5 min and then destained (3 x 5 min) in MeOH:H_2O (1:1).

The band of blotted protein was excised from membrane and sequenced using an Applied Biosystems Model 477A protein sequencer (Perkin Elmer, Foster City,

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CA). The first 21 amino acids were determined and compared to the protein library in the SwissProt Database. The sequencing services were generously provided by Dr. Michael Rohde at Amgen (Thousand Oaks, CA).

Fluorometric Triglyceride Lipase Bioassay

To determine if the activity of the putative enzyme was specific to phospholipids or non-specifically acting as a lipase at any ester linkage, the protein was screened in a lipase assay developed by Salvayre *et al.* 1986. The activity of the *Limulus* protein was compared to porcine pacreatic lipase (positive contol)(Sigma, St. Louis, MO), and no protein (blank). The assay consists of a triolean/colipase substrate (Boehringer Mannheim Diagnositics, Germany) with the fluorescent triglyceride. We were unable to obtain the original fluroescent substrate (1-pyrenedecanoyl-2,3-dioleyl glycerol) but substituted 1,3-dioleoyl-2[4-pyrenyl-butanoyl]-glycerol (2P4TG). The substrate was kindly provided by Dr. Paavo Kinnunen (Univ. of Helsinki, Finland). The lypholized triolean/colipase substrate was reconsituted with 20 ml DI and mixed gently. Fifteen pmol of 2P4TG, in hexane, was added to a vial, blown dry, reconstituted with 1 ml of triolean/colipase substrate and sonicated 4 x 15 sec. A 90 µl aliquot of this labeled substrate was added to 10 ul of enzyme. The tubes were heated at 37°C for 10 min and the reaction was stopped with 1.5 ml solvent (chloroform:MeOH:heptane, 125:140:100, v/v/v) and 0.5 ml of 0.5 M carbonate buffer, pH 10.5. The samples were centrifuged and the upper aqueous layer was measured on a Perkin Elmer LS 50B Fluorometer at

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excitation (342 nm) and emission (395 nm). The lipase activity for was calculated as U/L:

$$\text{Lipase, U/L} = \frac{F}{F_s} \cdot \frac{ED}{t} \cdot 10 .$$

where F = fluorescence of 4-pyrenylbutanoic acid (P4) liberated (corrected for subtraction of the blank). F_s = fluorescence of 1 pmol of P4 as measured under assay conditions. ED = enzyme dilution and t = time (min).

General Inhibition Studies

General comparisons of the PLA_2 activity were drawn between the *Limulus* protein and $rhPLA_2$ by screening the *Limulus* protein under a variety of conditions. The *E. coli* assay, as previously described, was used to measure the level of PLA_2 activity. The four treatments for the *Limulus* protein were (1) heating for 10 min at 90-100°C alone (considered non-denaturing) or (2) in the presence of the reducing agent, ß-mercaptoethanol (denaturing conditions) (Davidson and Dennis 1990a), and (3) using the inhibitors manoalide (1 µM final conc.) or (4) bromophenacylbromide (1 µM final conc.) (Mayer and Marshall 1993).

Specific Manoalide Inhibition Studies

In order to compare the phospholipase A_2 activity in *Limulus* protein to that of the $rhPLA_2$, manoalide inhibition studies were carried out using the previously detailed *E. coli* procedure. Manoalide is a marine natural product isolated from the sponge *Luffariella variabilis* (Da Silva and Scheuer 1980). This compound is a potent

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covalent irreversible inhibitor of both secreted and cytosolic PLA_2s (Mayer and Marshall 1993). In the secreted phospholipases, manoalide functions by reacting with two lysine residues in the enzyme (Bianco *et al.* 1995, Lombardo and Dennis 1985).

The volumes and incubation times of the *E. coli* assay were adjusted to accomodate the use of an inhibitor. The variations in sample incubation times with substrate for crude versus a purified protein were recommended in Marshall *et al.* (1994). Triplicate samples were run for each manoalide concentration with both enzymes. For the 14-kDa-rhPLA_2 analyses: 10 µl enzyme (0.5 ng/µl), 25 µl reaction buffer and 5 µl of varying concentrations of manoalide in dimethylsulfoxide (DMSO) were combined and incubated for 30 minutes at room temperature. Then 10 µl *E. coli* suspension was added and the samples were incubated for 15 minutes at 37°C. Reactions were stopped and treated as previously described. For the *Limulus* protein analyses: 6 µl protein (9.1 µg/ml), 4 µl buffer (300 mM NaCl, 50 mM NaAcetate, BSA 1 mg/ml at pH 4.5), 25 µl reaction buffer and 5 µl of varying concentrations of manoalide (in DMSO) were combined and incubated for 30 minutes at room temperature. Then 10 µl of *E. coli* suspension was added and samples were incubated for 1 hr. at 37°C. The reaction was stopped and treated as previously described.

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Hemagglutination Assays

A standard hemagglutination assay for lectins was used (Brooks *et al.* 1997). Human red blood cells (4% suspension, v/v) of blood type A, B and AB were obtained from Sigma (St. Louis, MO). The neuraminidase and glutaraldehyde treated cells were shipped in phosphate buffered saline (PBS). Half of the cell suspension was left in the PBS solution and the remaining cells were washed and resuspended at 4% in lectin buffer (50 mM Tris Base, 150 mM NaCl, 1 mM $MgCl_2$, 0.8 mM $CaCl_2$, pH 7.6).

A 100 μl aliquot of the blood cell suspension was pipetted onto a microscope slide and 20μl of lectin solution was added to the cells. A negative control using only lectin buffer was used for comparison to samples. Commercially purified lectins from *Limulus polyphemus*, limulin, and from *Pseudomonas aeruginosa* strain A1 (PA-A1), (Sigma, St. Louis) were used as positive controls. The *Limulus* protein and PA-A1 lectin were tested by addition of 20 μl of 100 μg/ml and/or 5 μg/ml to the cells suspension, 16.6 ug/ml and 0.83 μg/ml final conc., respectively. The limulin was only tested at 5 μg/ml (0.83μg/ml final conc.). Agglutination was gauged by observing the red blood cells (RBC) under microscope (40X) at 5 minute intervals and recording the level of agglutination at 30 minutes.

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4.3 Results

Protein Isolation

Separation of the homogenate on the SP-Sepharose cation-exchange column yielded four major protein peaks. There was PLA_2 activity associated with two areas, one corresponding to the fourth protein peak and the other was associated with the small amount of protein eluting after the fourth peak (Figure 20). The active fractions from both areas were pooled and concentrated separately. An attempt to purify the activity eluting after the fourth peak ended after total loss of all activity during further purification steps. The activity associated with the protein peak was fully characterized. The first column gave a 2-fold purification and a 10% yield. A drop in PLA_2 activity was seen after the Sepharose column (Table 8), but this was similar to the human synovial PLA_2, so it was not unexpected (Kramer and Pepinsky 1991).

The separation of the Sepharose concentrate on the Sephadex G-50 size exclusion column revealed two protein peaks (Figure 21). The majority of the biological activity was associated with the second protein peak. After concentration and protein estimation, the Sephadex column gave a 19% yield and a 14-fold purification of the enzymatic activity.

The separation of the Sephadex concentrate on the semi-preparative RP-HPLC revealed two protein peaks which coeluted (Figure 22). The phospholipase A_2 activity was associated with both peaks due to the coelution. The active fractions were pooled and concentrated for further characterization. After this final purification step there was a 142-fold purification and a 34% yield of protein (Table 8).

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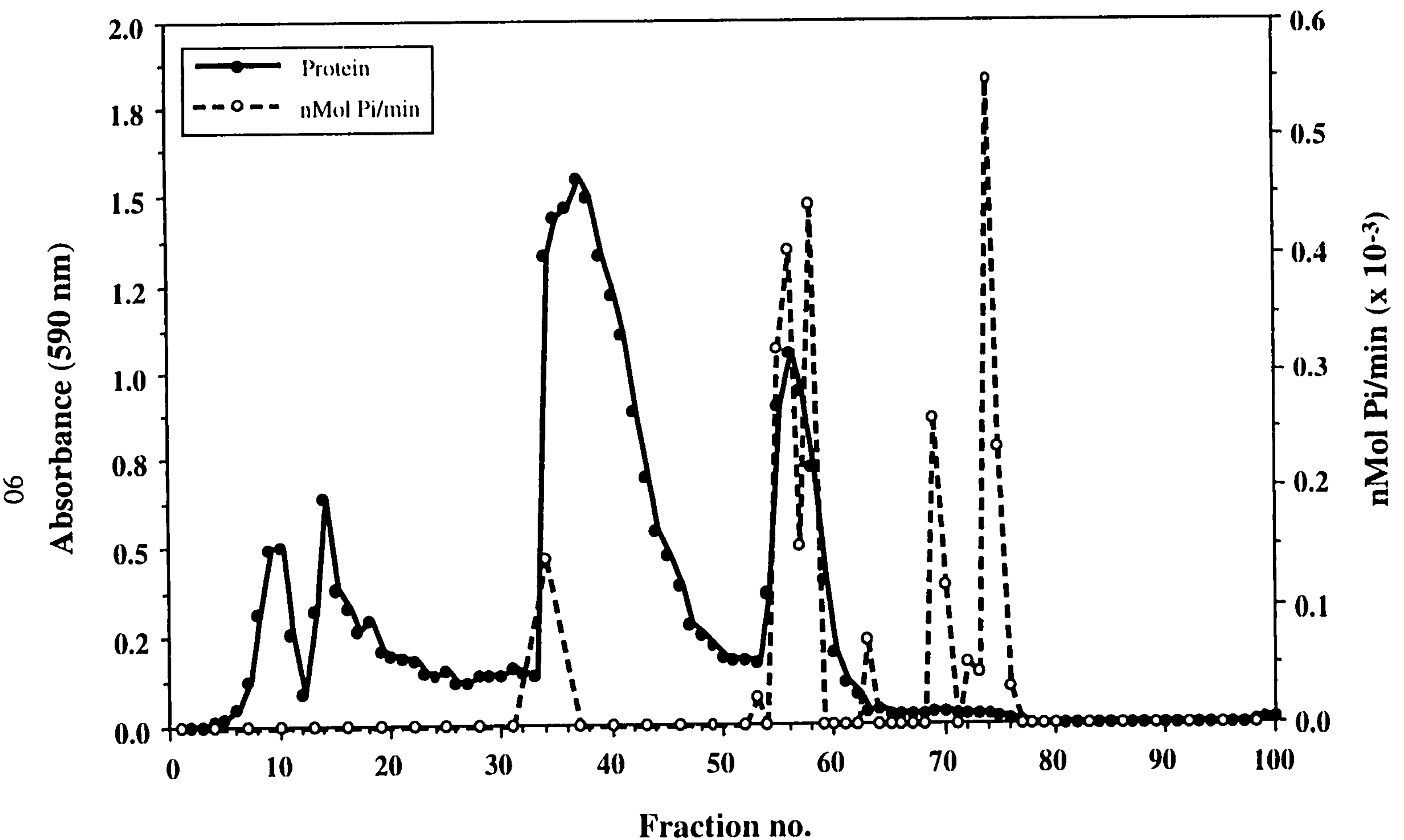

Figure 20. Protein elution and PLA_2 activity profiles of the amebocyte crude homogenate separated on a SP-Sepharose Fast Flow cation-exchange column.

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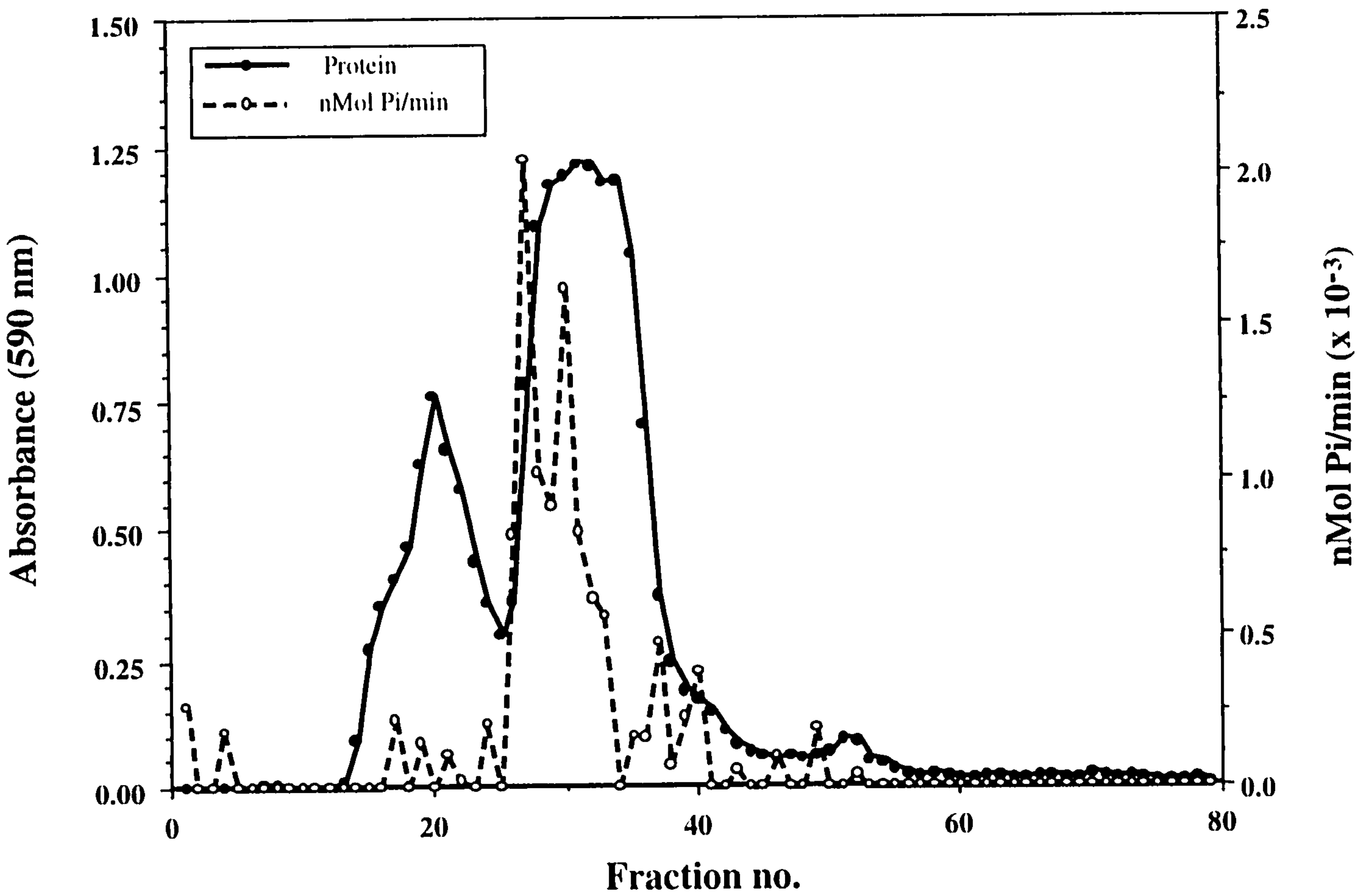

Figure 21. Protein elution and phospholipase A_2 activity profiles of the Sepharose concentrate separated on Sephadex Superfine G-50 size-exclusion column.

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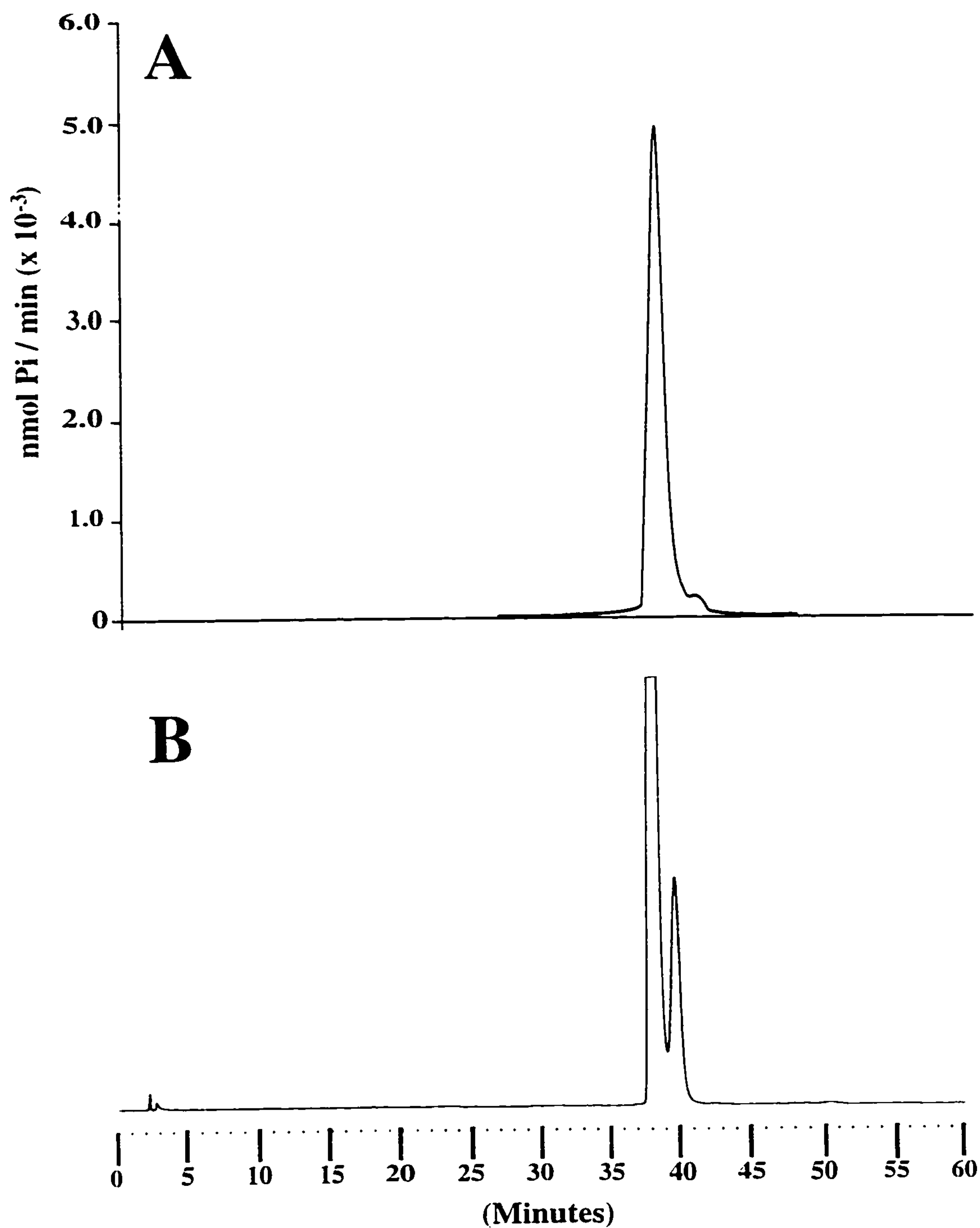

Figure 22. Reversed-phase HPLC separation of the Sephadex concentrate A) phospholipase A_2 activity monitored by *E.coli* assay and B) the protein elution profile.

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Table 8. Purification table of the putative phospholipase A_2 from *Limulus* amebocytes.

	Protein (mg)	Units (nmol/min)	Units/mg	Yield (%)	Purification (-fold)
Crude Extract	2133	8.61	0.004	100	
SP-Sepharose	95.4	0.87	0.009	10	2
Sephadex G-50	28.6	1.61	0.056	19	14
RP-HPLC	19.2	2.96	0.573	34	142

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Protein Characterization

The SDS-PAGE analysis showed the isolation of a predominant protein band just below 21.5 kDa (Figure 23). After the Sepharose column, the majority of protein bands present were below 31 kDa and a strong band predominated between 14.5 and 21.5 kDa. After the Sephadex column, a few minor protein bands remained and the two major bands left were below 21.5 kDa. The separation of these proteins was not greatly enhanced by the semi-preparative RP-HPLC column (Fig. 23; lanes 3-4).

The RP-HPLC concentrate was subjected to Western blot analysis using the polyclonal recombinant human PLA_2 antibody (Figure 24). Only one band was reactive with the type II 14-kDa recombinant human PLA_2. The negative controls, lysozyme and limulin, demonstrated negligable antibody reaction. After comparing the gel (lane 4) and the blot, it is evident that the protein displaying cross-reactivity with the $rhPLA_2$ is the main band just below 21.5 kDa.

The ESI-MS profile of the centroid analysis of the RP-HPLC concentrate revealed four proteins estimated at 6/7 kDa, 12 kDa, 18.5 kDa and 24.5 kDa. The 12 and 18.5 kDa proteins are in a comparable size range to the type II 14-kDa phospholipase (Marshall and Mayer 1993). The larger protein, which is the main reactive band in the Western blot, was reanalyzed in continuum mode and the second analysis produced an protein envelope which deconvoluted to give an accurate mass estimate of 18499 Da (Figure 25).

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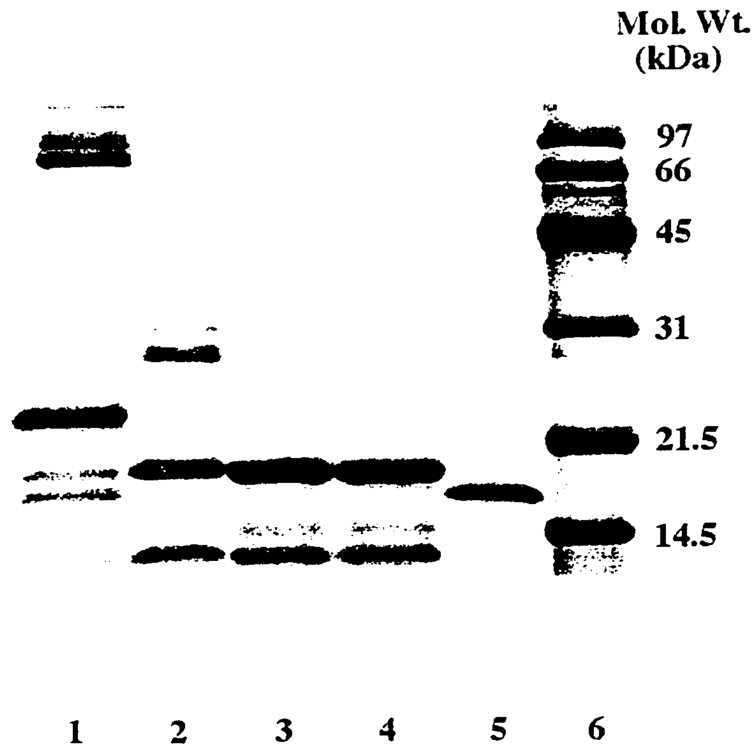

Figure 23. SDS-polyacrylamide gel electrophoresis separation (15% Tris-glycine) of proteins at each stage of the protein isolation [1. crude extract; 2. SP-Sepharose column; 3. Sephadex G-50 column; 4. RP-HPLC column; 5. Recombinant human PLA_2; 6. Mol. Wt. Stds].

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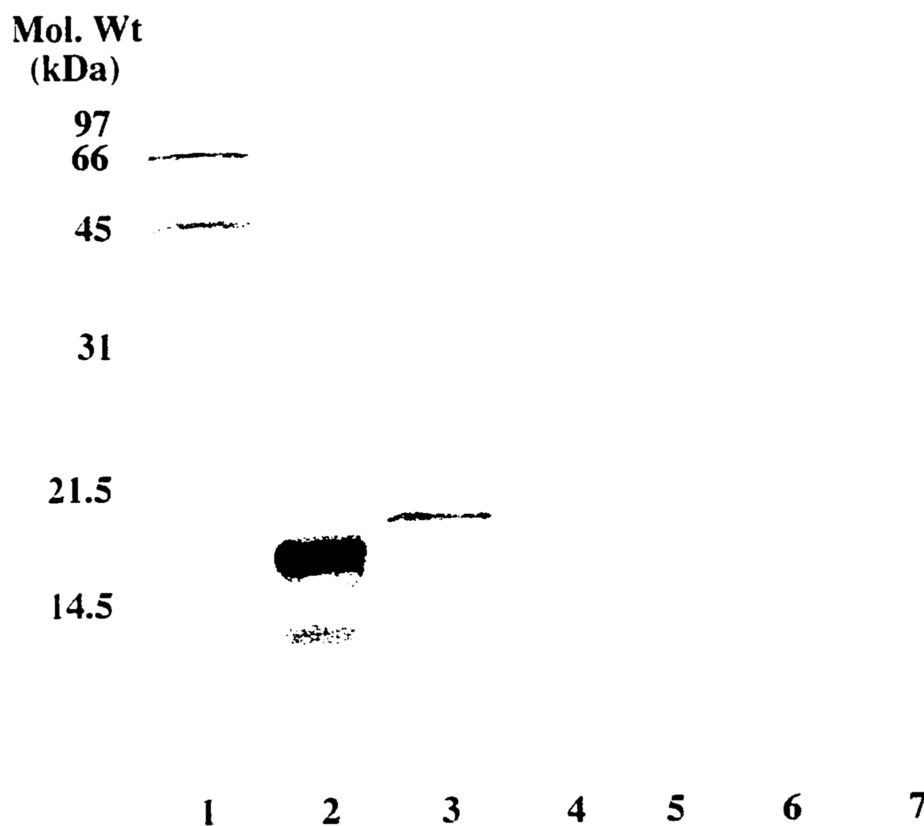

Figure 24. Western blot of the *Limulus* putative PLA_2 against recombinant human 14-kDa PLA_2 using a biotinylated alkaline phosphatase detection system. [1. Mol. wt. std.; 2. Recombinant human PLA_2; 3. RP-HPLC concentrate; 4 and 5. Empty; 6. Lysozyme; 7. Limulin]

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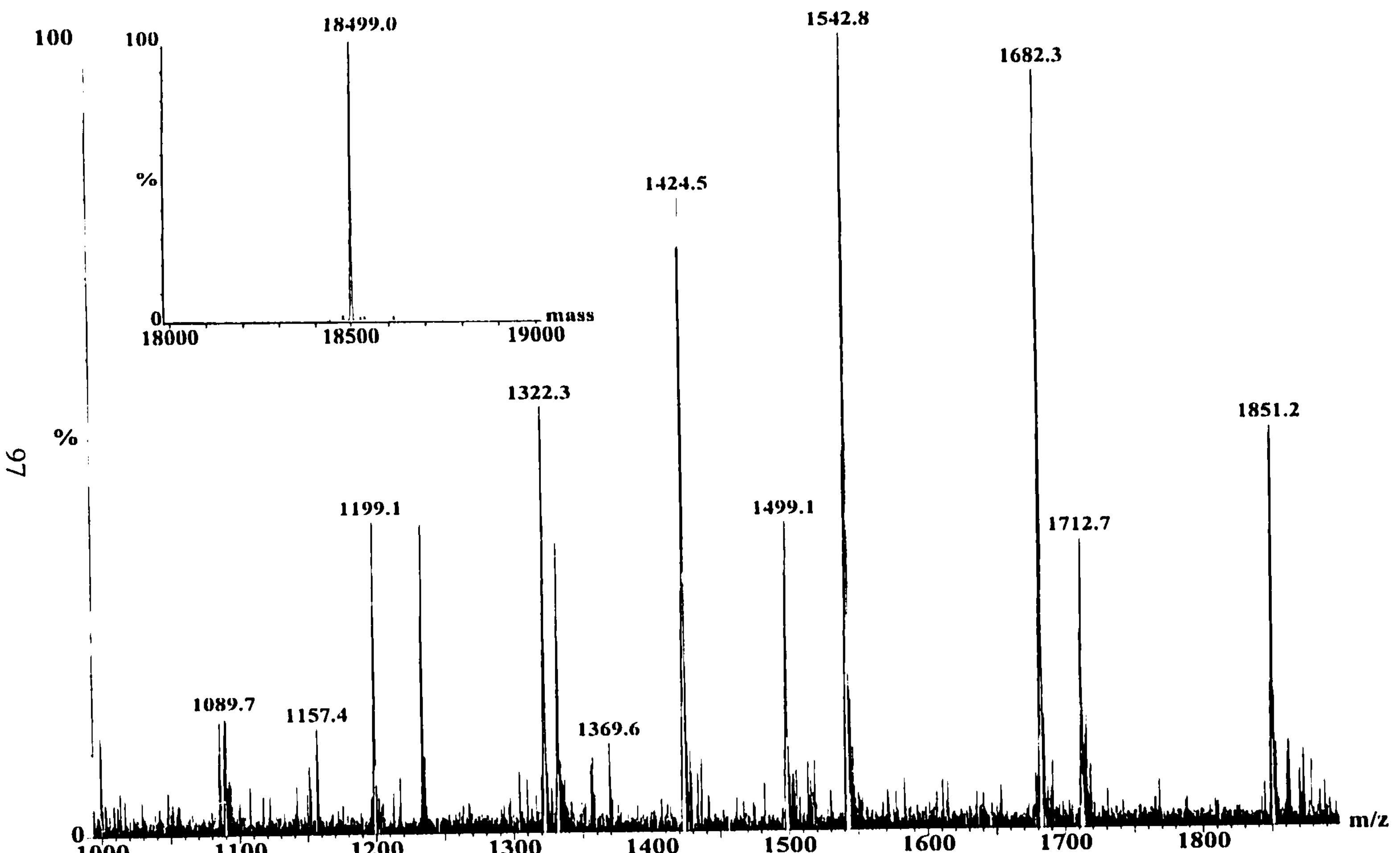

Figure 25. Protein envelope and deconvoluted mass estimate (insert) of the 18.5 kDa *Limulus* protein using positive-ion electrospray ionization-mass spectrometry.

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The partial sequence of the protein yielded twenty of the first twenty-one amino acids (Table 9). This partial sequence was submitted to the SwissProt Database and was found to almost perfectly match a previously published sequence of a *Limulus* protein defined as a hemagglutinin (Fujii *et al.* 1992).

The general phospholipase activity of the *Limulus* protein was compared to that of rhPLA$_2$ after the *Limulus* protein had been boiled alone or in the presence of ß-mercaptoethanol (ß-ME), as well as in the presence of known PLA$_2$ inhibitors, bromophenacylbromide (BPB) and manoalide (MLD) (Figure 26). The *Limulus* protein only lost a small amount of activity after boiling. It has been demonstrated that small secreted PLA$_2$s do not lose much of their activity, even after prolonged heating, because the multiple disulfide bonds lead to a reversal of denaturation upon cooling (Mayer and Marshall 1993, Davidson and Dennis 1990a). The five percent drop in activity after boiling the *Limulus* protein is similar to that seen in cobra venom PLA$_2$ (Davidson and Dennis 1990a). The addition of ßME, while heating, abolished all enzymatic activity. The level of the PLA$_2$ activity was reduced in the presence of both BPB and MLD, though in the presence of MLD the reduction in the *Limulus* protein was not as dramatic as that seen in the rhPLA$_2$.

The phospholipase A$_2$ activity of the *Limulus* protein was inhibited in a dose dependent fashion in the presence of varying concentrations of the competitive irreversible inhibitor manoalide (Figure 27). The slopes of the two regression lines indicate that the rate of susbstrate cleavage by the *Limulus* protein is about 3-fold lower than that of rhPLA$_2$. The rhPLA$_2$ was also more sensitive to the inhibitor and its enzymatic activity was abolished at about 50µM MLD, whereas for the *Limulus*

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Table 9. Partial sequence comparison of the 18.5 kDa *Limulus* protein from the PVDF blot with the previously described *Limulus* hemagglutinin SwissProt Database sequence.

18.5 kDa	**W**	**V**	**N**	**D**	**W**	**D**	**G**	**A**	**L**	**N**	**F**	**Q**	**X**	**Q**	**L**	**K**	**D**	**S**	**I**	**K**
Haal_f*	W	V	N	D	W	D	G	A	L	N	F	Q	C	Q	L	K	D	S	I	K
	1									10										20
	T																			
	T	I	S	S	I	H	S	N	H	H	E	D	R	R	W	N	F	G	C	E
										30										40
	R	T	L	R	D	P	S	C	Y	F	T	N	Y	V	N	D	W	D	K	L
										50										60
	L	H	F	T	C	K	S	G	E	A	I	A	G	F	N	S	Y	H	D	N
										70										80
	R	R	E	D	R	R	W	K	I	Y	C	C	K	D	K	N	D	L	H	Y
										90										100
	T	K	C	T	D	Y	R	T	C	A	W	T	G	Y	V	N	S	W	D	G
										110										120
	V	P	K	D	Y	V	L	T	G	V	I	S	E	H	D	N	H	R	E	D
										130										140
	R	R	W	K	F	Q	H	C	R	L	K	N	C							
										150										

* SwissProt Database designation for the *Limulus* hemagglutinin.

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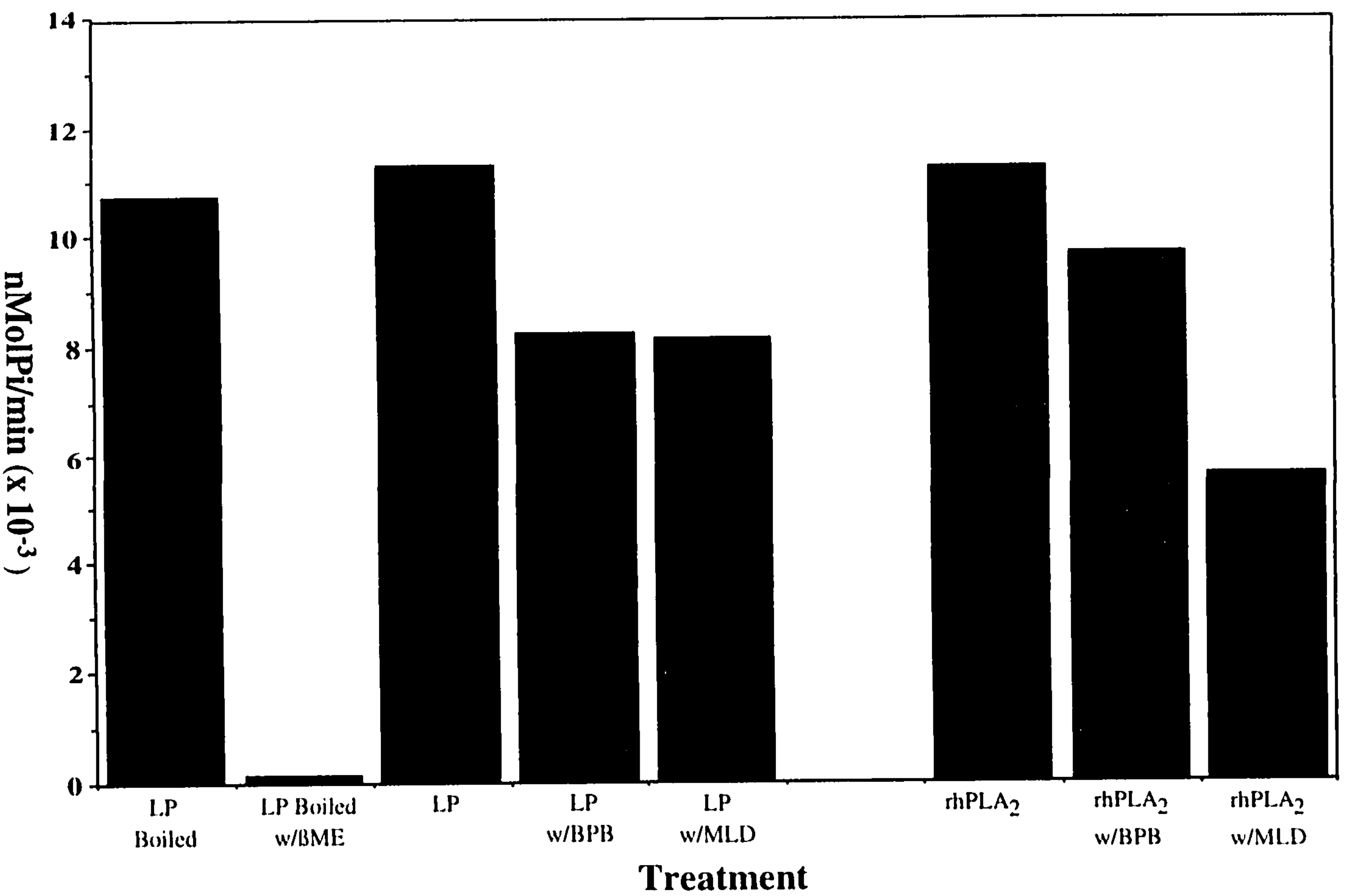

Figure 26. Comparison of phospholipase A_2 activity of the putative *Limulus* PLA_2 and recombinant human 14-kDa PLA_2 ($rhPLA_2$) alone and in the presence of inhibitors [ßME=ß-mercapto-ethanol, BPB = bromophenacylbromide (1μM) and MLD = manoalide (1μM)].

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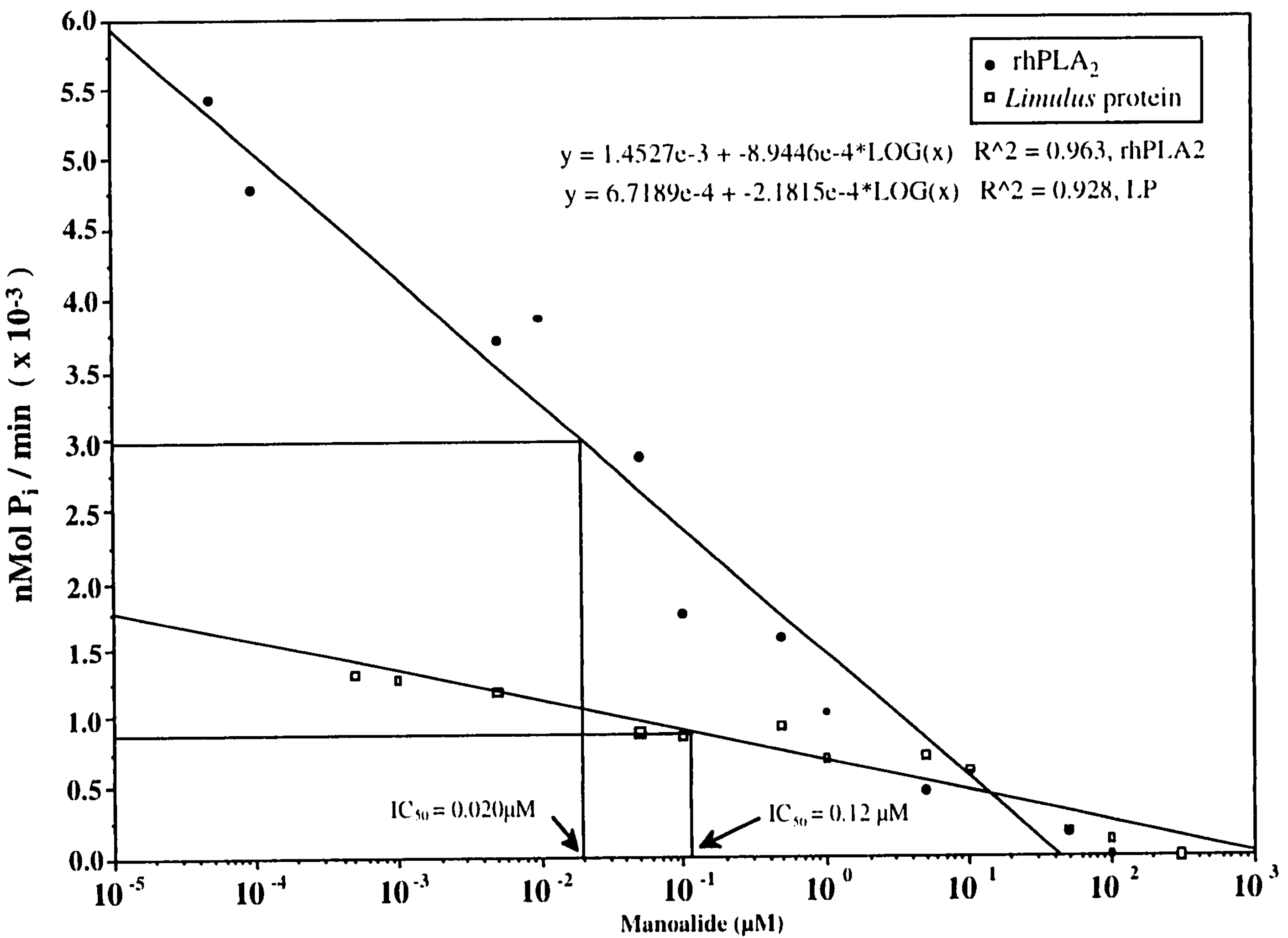

Figure 27. Comparative enzymatic activity analyses of recombinant human PLA_2 and *Limulus* protein in the presence of the irreversible inhibitor manoalide.

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protein at least 300 μM MLD was required. The concentration where 50% inhibition occurred (IC_{50}) in the rhPLA2 was 0.020 μM, which agrees well with the published IC_{50} of 0.017 μM for this enzyme using the *E. coli* assay in the presence of manoalide (Mayer and Marshall 1993). The IC_{50} for the *Limulus* protein was 6-fold greater than the $rhPLA_2$ at 0.12μM MLD.

The biological activity of the *Limulus* protein was compared with porcine pancreatic lipase. A lipase can cleave an ester linkage regardless of its location in either triglycerides or phospholipids, but a phospholipase will not cleave a triglyceride ester linkage. The *Limulus* protein displayed no activity in the lipase assay and levels of 4-pyreneylbutanoic acid release were below the level of detection (Table 10).

The agglutinating activity of the *Limulus* protein was compared to limulin, a 55 kDa lectin from *Limulus polyphemus* plasma, and a bacterial lectin from *Psuedomonas aeroginosa* strain A1 (PA-A1). The results from these assays were not straight forward (Table 11). The PA-A1 lectin was most effective in group A RBCs and at either 100 μg/ml or 5 μg/ml levels there was major or total agglutination of all cells as expected (Sigma specs.). In group B and AB RBCs, only the 100 μg/ml concentration of PA-A1 induced total agglutination, though at 5 μg/ml at least 30-70% of cells agglutinated. Limulin should be more effective at agglutinating group B cells (Sigma specs.) though the lectin buffer, which provides the appropriate pH and Ca^{2+}/Mg^{2+} levels (Brooks *et al.* 1997), seemed to inhibit the agglutination. The *Limulus* protein displayed some activity at 100μg/ml, but at 5 μg/ml agglutination levels were similar to that in the negative controls. The *Limulus* protein displayed the greatest activity, 20-30%, in Group A and AB RBCs at 100 μg/ml. This level of

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Table 10. Lipase activity of the *Limulus* protein in comparison to pancreatic lipase using a fluorometric lipase assay.

Enzyme	Fluorescence Units	Activity (U/L)
Pancreatic Lipase	29.016	262500
	17.868	131250
No enzyme	5.255	0
Limulus protein	4.128	ND
Limulus protein (1:10)	4.217	ND

n=4, except 131250 U/L
ND = not detectable, less than zero

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Table 11. Percent hemagglutinin activity in two purified commercial lectins and the *Limulus* protein after a thirty minute incubation of each lectin with a 4% suspension of human red blood cells.

Sample	Human Group A		Human Group B		Human Group AB	
	LB*	PBS †	LB	PBS	LB	PBS
P. aeruginosa PA-1 lectin (100 μg/ml)	100	100	100	100	100	100
P. aeruginosa PA-1 lectin (5 μg/ml)	70-80	100	60-70	50	50-60	30-40
Limulin (5 μg/ml)	20-30	20-30	5-10	30-40	5-10	30-40
Limulus Protein (100 μg/ml)	20-30	5-10	10-20	10	10	20-30
Limulus Protein (5 μg/ml)	5-10	10-20	2-5	10	10	10-15
Buffer Control	5-10	5-10	10-15	10	10	10-15

* LB = lectin buffer
† PBS = phosphate buffered saline

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agglutination was only twice that of control levels and less than or equal to levels seen using limulin. It is difficult to compare these results to those previously published using heparinized horse erythrocytes (Fujii *et al.* 1992). Assays were attempted with lyophilized horse erythrocyes (Sigma), but after resuspension in either PBS or lectin buffer no agglutination activity was detected even after an hour incubation with PA-A1. The horse RBCs were considered non-viable and not used for further testing.

4.4 Discussion

This final chapter culminates with the sucessful isolation of a putative PLA_2 from calcium ionophore and arachidonic acid stimulated *Limulus* amebocytes. After a partial sequence was acquired for this protein, a comparison to the SwissProt Database revealed that the 18.5 kDa protein had been previously described by Fujii *et al.* (1992) and was defined as an agglutinin / aggregation factor. The authors isolated the protein, using an agglutination assay to detect biological activity. They compared the protein sequence by FASTA to all known proteins and results indicated a 37% sequence similarity to the tryosine-rich acidic matrix protein (TRAMP), bovine dermatopontin, which participates in collagen fibril formation (MacBeath *et al.* 1993). In the thesis work carried out by this researcher, the *Limulus* protein clearly reacted in a well-established phosopholipase A_2 assay and demonstrated some specificity by displaying no activity in a general lipase assay. The protein also gave a positive cross-reaction to polyclonal $rhPLA_2$ antibody in a Western blot while negative controls, lysozyme and limulin, gave a negligible responses. The PLA_2 activity was inhibited in a dose-dependent fashion by the irreversible PLA_2 inhibitor, manoalide, as well as in the presence of BPB which binds to histidine at the active site of PLA_2s.

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The *Limulus* protein did not lose activity after heating alone, but activity was abolished after heating in the presence of ßME. Also, this protein is stored in the L-granules of the amebocyte and Rosenthal *et al.* (1995) demonstrated that the group II PLA_2 in neutrophils is stored in the cells' granules. So, there are arguments both in favor and against the hypothesis that this protein is potentially a phospholipase A_2.

The *Limulus* protein did display limited agglutinating activity and it is difficult to reconcile the phospholipase A_2 activity given the general definition of agglutinins, also termed lectins. Lectins should (1) have two or more sugar binding sites, (2) not act as antibodies, (3) not possess enzymatic activity and (4) be distinct from certain toxins (i.e. ricin) (Brooks *et al.* 1997, Goldstein and Poretz 1980). If this protein possesses enzymatic activity, than it cannot be a true lectin. This protein is secreted and though many C-type lectins are extracellular or plasma membrane associated, there are some low molecular weight secreted lectins (group VII) such as the pancreatic stone protein (Gabius 1997, Drickamer and Taylor 1993). But, it should be also noted that PLA_2s from snake and bee venoms have been associated with lectin-like activity (Ouyang *et al.* 1992, Berezin *et al.* 1997).

In their assay system, Fujii *et al.* (1992) indicated agglutination of the heparanized horse RBCs occurred at concentrations as low as 10 ng/ml. When the *Limulus* protein agglutinating activity was tested in neuraminidase and glutaraldehyde treated human RBCs with 100 µg/ml (17 µg/ml final conc.) and 5 µg/ml (0.8 µg/ml final conc.) minimal agglutinating activity (avg. 15%) was detected. This is curious since pretreatment of RBCs with neuraminidase, or enzymes such as papain or trypsin, typically sensitizes the cells to agglutination (Goldstein and Poretz 1980).

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When Fujii *et al.* (1992) isolated this protein they stated they performed the agglutination assay "..as essentially described.." by Bishayee and Dorai (1980), but they made one major modification, they heparinized the horse erythrocytes, whereas the original method simply used washed rabbit erythrocytes. Heparin is commonly used in the isolation of platelets for platelet aggregation studies, but aggregation in platelets is chemically induced by the release of endogenous adenosine diphosphate after cell activation. In hemagglutination assays, the lectins bind to sugars on the surface of the cells and the lectin has two binding sites allowing it to bind sugars on separate cells triggering agglutination.

There is a possible alternative explanation for the reaction seen in the agglutination assays performed by Fujii *et al.* (1992). That is, by treating the RBCs with heparin they inadvertently created the optimal conditions for this protein to bind to the RBCs. This explanation is based on the premis that low molecular weight non-pancreatic secreted PLA_2s have a high affinity for heparin and heparin-Sepharose affinity chromatography has been used to purify a number of secreted PLA_2s from human synovial fluid, rat peritoneal fluid and activated rat and rabbit platelets (Hara *et al.* 1991).

As previously stated, Fujii *et al.* (1992) found that this protein shared 37% overall identity with the TRAMP protein, bovine dermatopontin. When this researcher ran a FASTA of the *Limulus* protein against the SwissProt Database, it indicated only a 26.5 % similarity in a 162 amino acid overlap to bovine dermatopontin. The FASTA search also indicated some similarity to human dermatopontin, 28.1%, but not to any known lectins. When making simple sequence

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comparisons. Doolittle (1995) stated that "as a rule of thumb" the cut-off point to safely identify two proteins that have diverged from a common ancestor is 30%. According to the more recent FASTA, the *Limulus* and dermatopontin proteins are below this level. The dermatopontins belong to the TRAMP family and though the 10 half cysteine residues of the *Limulus* protein and the dermapontin remain conserved, the authors themselves point out that the *Limulus* protein lacks of the key N-terminal TRAMP region and that there is a disparity between the two proteins pIs (pI = 4.6, dermapontin and 8.3, *Limulus* protein). The range published for some of the group II PLA_2s is basic at 8.3-9.3 (Cupillard *et al.* 1997). Therefore, the argument that the *Limulus* protein is related to dermatopontins is now somewhat weak.

Fujii *et al.* (1992) also promote the importance of the short repeat, Glu-Asp-Arg-Arg-Trp found three times in the *Limulus* protein. At the comparative sites in the dermatopontin protein, there is retention of the Glu-X-Asp-X-Trp, but the X positions are variable at each site. There is a danger in comparing small sequences in the primary sequence data between two proteins. Doolittle (1994) points out that short sequence comparisons, five to six amino acids, could seem to imply convergence, but longer stretches of the protein usually demonstrate a divergence which negates chance identities. The authors also make reference to the lack of Arg-Gly-Asp sites in both proteins, which is common in C-type selectins. But the *Limulus* protein has two HDN (His-Asp-Asn) groups, and the His-Asp pair is conserved in all active secreted PLA_2s (Davidson and Dennis 1990a, Dennis 1997).

The *Limulus* protein also lacks primary sequence similarity to published PLA_2s. The low molecular PLA_2s usually display high levels of overall similarity

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(~40%) within their particular groups and almost complete similarity at the active site (Dennis 1997, Heinrikson 1991, Davidson and Dennis 1990b). Phylogenetic comparisons between PLA_2s, as well as to a theoretical ancestor PLA_2s, have been carried out on groups I-III PLA_2s. There was a demonstrated conservation of the cysteines, though the large gaps and deletions required to compare group III to groups I and II could invalidate this comparison (Davidson and Dennis 1990b, Maraganore *et al.* 1987). Until 1989, only snake venoms and pancreatic PLA_2 were known, but the super family has has ballooned to nine groups, two with multiple subgroups and primary sequence comparisons without consideration of native protein structure and *in vivo* activity are being questioned (Tischfield 1997). This agrees with Doolittle (1994) whose review documents that completely different folds and sequence arrangements can result in similar function when the same geometry is formed in the quaternary structure. Therefore, the three dimensional definition of the catalytic site in a protein can outweigh the disparity in primary sequence comparisions.

The growing super family of PLA_2s includes a new unrelated PLA_2 from the venom of the marine snail, *Conus magus*. This PLA_2 displays virtually no sequence similarity though it is a 13.6 kDa enzyme with 6 disulfide bonds and a His-Asp pair (Dennis 1997, McIntosh *et al.* 1995). The *Limulus* protein contains 5 disulfide bonds and two His-Asp pairs. The molecular weight, number of disulfide bonds and His-Asp pairs are most similar to that seen for type III secreted PLA_2s from bees and lizards (16-18 kDa, 5 disulfides and a His-Asp pair). The discovery of the *Conus* venom is accompanied by the recent documentation of PLA_2 activity other invertebrates and lower organisms. Phospholipase A_2 activity has been reported now

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from the tobacco hornworm, *Manduca sexta* (Uscian and Stanley-Samuelson 1993); an ant *Pseudomyrmex triplarinus* (Hinks *et al.* 1994); the lone star tick, *Amblyomma americanum* (L.) (Bowman *et al.* 1997); a jellyfish, *Rhopilema nomadica* (Gusmani *et al.* 1997), as well as the protozoan *Tetrahymena pyriformis* (Kovács and Csaba 1997).

A final note on the cross-reactivity of the *Limulus* protein in the Western blot. Antibodies recognize epitopes, or antigenic determinants, on macromoleclues. Epitopes can be either sequential, where there are several contiguous amino acid sequences within a given protein, or non-sequential, where the primary amino acid sequence of the binding sites are quite different. It has been established with the type III bee venom PLA_2 that both monoclonal and polyclonal human antibodies raised to the enzyme recognize discontinuous epitopes (Schneider *et al.* 1994). Though it should be noted that non-seqential epitope binding can be disrupted by reducing the disulfide bonds in the protein (Kuby 1997). Also immunization with adjuvant is often accompanied by denaturation of the antigen, thereby allowing polyclonal antibody formation to sites other than those available on the native protein (Scibienski 1973), but the negligable response seen in the negative controls strengthens the argument that the cross-reaction is valid.

This researcher believes that the biological evidence that this protein has phospholipase A_2 activity is convincing, but considering the lectin-like and aggregating activities associated with some PLA_2s the ability of this protein to promote aggregation cannot be totally discounted at this time.

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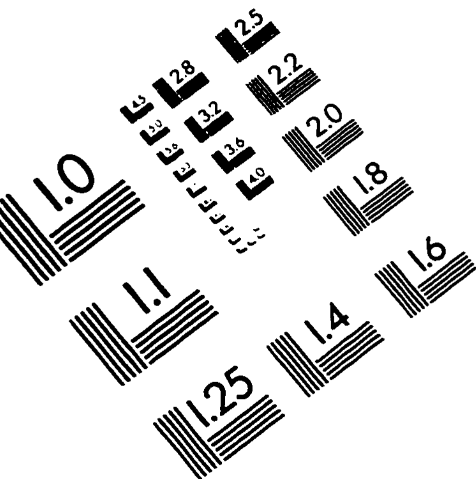
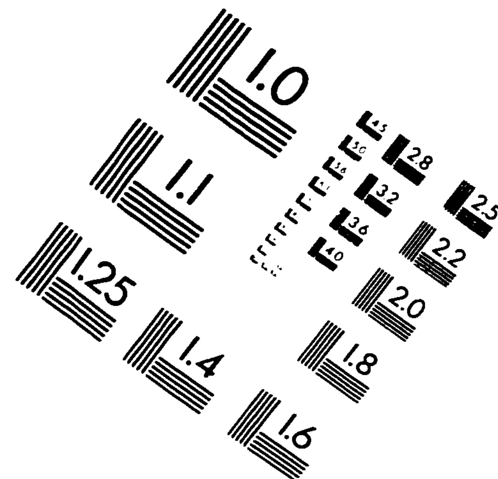
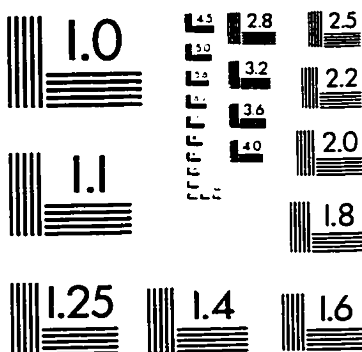
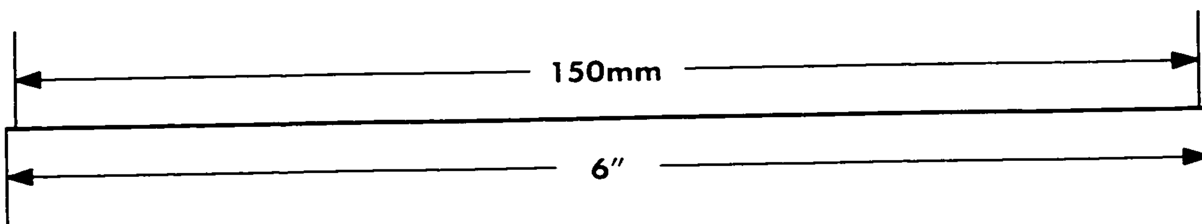
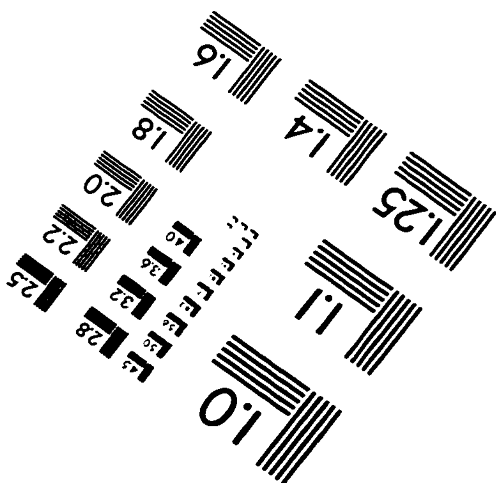
IMAGE EVALUATION TEST TARGET (QA-3)

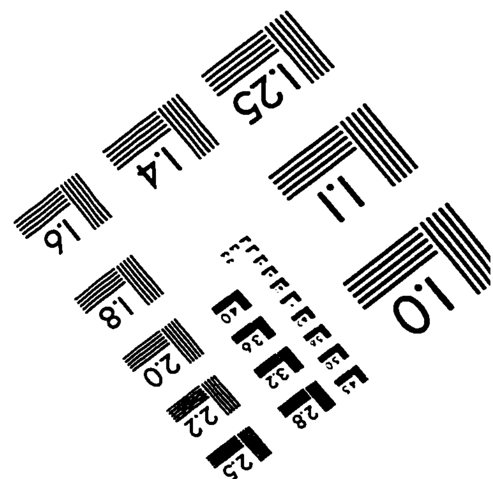












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