THE OCCURRENCE, DISTRIBUTION, AND REMODELING OF ARACHIDONIC ACID IN PHOSPHOLIPID FRACTIONS FROM THE SALIVARY GLANDS OF AMBLYOMMA AMERICANUM (L.)

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INTRODUCTION

Each part of this thesis is a separate and complete manuscript to be submitted for publication. Part I is being published in Archives of Insect Biochemistry and Physiology. Part II has been submitted to Journal of Parasitology. Part III is being submitted to Insect Biochemistry and Molecular Biology. Each part is presented in the thesis in the format of the journal to which it is submitted.

PART I

ANALYSIS OF LIPIDS IN THE SALIVARY GLANDS OF *AMBLYOMMA AMERICANUM* (L.): DETECTION OF A HIGH LEVEL OF ARACHIDONIC ACID

ABSTRACT

Analysis of lipids in salivary glands of the lone star tick, Amblyomma americanum, demonstrated that arachidonic acid (20:4, n-6) comprises 8% of all fatty acids identified by gas chromatography. The occurrence of arachidonic acid and other C_{20} polyunsaturated fatty acids in tick salivary glands was confirmed by gas chromatography-mass spectrometry. Arachidonate is located entirely in the phospholipid fraction and is associated exclusively with phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Salivary glands stored and frozen for several months had a similar lipid composition as freshly dissected salivary glands, with the exception of a small amount of free arachidonic acid and an increase in lysophosphatidylcholine. Incubation of salivary gland homogenates with snake venom phospholipase A_2 showed that most saturated fatty acids are esterified in the sn-1 position of PC and PE, with the unsaturated fatty acids in the sn-2 position. Approximately 75% of arachidonic acid is in the sn-2 position of PC and PE, adding support to the hypothesis that arachidonic acid is released into the cytoplasm after activation of a phospholipase A_2 for subsequent metabolism to prostaglandins and/or other eicosanoids.

INTRODUCTION

The salivary glands of ixodid ticks are sites for the development of infectious stages of various pathogens which may be co-secreted with saliva into the host during tick feeding [1]. Various components of tick saliva facilitate feeding. During penetration of the host skin, cement is secreted by the salivary glands to help firmly attach the mouthparts to the host [2]. An anticoagulant is secreted to keep the host blood fluid [3,4], and a carboxypeptidase in the saliva destroys inflammatory mediators such as bradykinin and anaphylatoxins [5].

Other molecules found in tick saliva include eicosanoids such as prostaglandins. Prostaglandins have been identified in the saliva or whole salivary glands of at least four species of ticks. Higgs et al. [6] and Dickenson et al. [7] first discovered PGE_2 in the saliva of the cattle tick Boophilus microplus. PGE_2 and $PGF_{2\alpha}$ were found by Shemesh et al. [8] in salivary gland homogenates of Hyalomma anatolicum. The saliva of Ixodes dammini was reported to contain PGE_2 [9] and prostacyclin [10]. More recently, PGE_2 and $PGF_{2\alpha}$ were identified in the saliva of the lone star tick, Amblyomma americanum [11]. Prostaglandin E_2, a potent vasodilator, may help maintain the host feeding lesion by dilating skin capillaries and

stimulating an increased blood flow to the mouthparts of the tick [3]. Tick derived eicosanoids may also prevent host hemostatic reactions, preclude activation of macrophages, and inhibit neutrophil activity [12].

The primary precursor of eicosanoids is arachidonic acid (20:4, n-6), which is often liberated from plasma membrane phospholipids into the cytoplasm through the activation of a phospholipase A_2 [13,14]. A phosphatidylinositol specific phospholipase C can also act on PI to generate a 1,2-diglyceride and inositol phosphate [15]. The resulting diacylglycerol may be a source of free arachidonic acid following cleavage by a diglyceride lipase or by a monoglyceride lipase in a two-step reaction sequence causing release of free fatty acids [16]. Once free in the cytoplasm, arachidonic acid is rapidly converted to prostaglandins by prostaglandin synthase [17].

Arachidonic acid in the phospholipid pool is necessary for eicosanoid synthesis, an event not extensively studied in arthropods. The purpose of present experiments was to assess the lipid composition of the salivary glands of an ixodid tick, A. americanum (L.) and more specifically to investigate the occurrence of and determine the amounts and location of precursor molecules needed for eicosanoid biosynthesis, such as arachidonic acid.

MATERIALS AND METHODS

Materials

Lipid and fatty acid methyl ester standards, phospholipase A_2 (from Naja naja), boron trifluoride, butylated hydroxytoluene, and other chemicals were acquired from Sigma, St. Louis, MO. L-3-Phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl was purchased from Amersham Corporation, Arlington Heights, IL. Thin-layer chromatography plates used were normal phase GHLF HPTLC plates: 10 x 20 cm, 250 μm thickness; and 20 x 20 cm preparative plates, silica gel GF plates; 500 μm thickness, acquired from Analtech, Newark, DE., and silica gel 60A preparative plates (LK6F plates); 20 x 20 cm, 250 μm thickness; from Whatman, Maidstone, England. Silicic acid (Bio-Sil A; 100-200 mesh) was purchased from Bio-Rad, Richmond, CA. Diethyl ether was obtained from EM Science, Gibbstown, NJ. All other solvents were from Fisher Scientific, Pittsburgh, PA, and were redistilled in glass before use.

Collection of Tissue

Lone star ticks, Amblyomma americanum (L.) were raised according to the methods of Patrick and Hair [18]. Adult

female ticks were placed on ovine hosts along with male ticks and allowed to feed for several days prior to removal. Unfed ticks were maintained at 27-28°C and 90-100% relative humidity before infestation of the host. In most experiments, salivary glands from partially-fed female ticks were dissected in 0.1 M MOPS buffer (pH 6.8) and analyzed for lipid content immediately without storage. In other experiments, the salivary glands were dissected in 0.1 M MOPS buffer and stored in 0.05 M PIPES buffer (pH 6.8), 20 mM EGTA and 40% glycerol at -15°C before use.

Extraction of Total Lipid

Salivary glands (20-40) were suspended in 800μl 0.1 M MOPS, pH 6.8, containing 20 mM EGTA, and in most experiments were homogenized in a glass centrifuge tube with a Tekmar SDT-1810 Tissumizer, Cincinnati, OH. In some experiments, salivary glands were macerated by hand in a ground glass homogenizer. An aliquot of 50μl of crude tissue homogenate was removed for analysis of total protein [19]. Lipids were extracted from the remaining homogenate by the methods of Bligh and Dyer [20] and stored in chloroform with BHT (5 mg/100 ml) to prevent oxidation.

Analysis of Fatty Acids in Total Lipid

Lipid samples were dried under N_2, and 50μl of a 1 mg/ml solution of heptadecanoic acid methyl ester (17:0) was added to each sample as an internal standard. Lipids

were hydrolyzed with 5% methanolic KOH at 60°C for 90 min and fatty acids were converted to methyl esters by the addition of boron trifluoride (14% in methanol) as described by Ryan et al. [21]. The chloroform extract containing fatty acid methyl esters was dehydrated by passing through a pipette containing a small amount of anhydrous $MgSO_4$ secured by a plug of glass wool. Solvent was removed under nitrogen, and samples were resuspended in 200 μl hexane and added to a pipette containing activated (at 100°C for 90 min) 100-200 mesh Bio-Sil A, which was washed with 3 ml hexane to remove hydrocarbons. The fatty acid methyl esters were then eluted from the column with 6 ml 5% diethyl ether in hexane. Fatty acids in the lipid extract were assessed by gas chromatography (Hewlett-Packard, Sunnyvale, CA, 5890 GC with flame ionization detector). Samples were run on three fused silica columns of differing polarities (DB-5, 30 m x 0.25 mm, 0.25 μm film thickness; DB-23, 30 m x 0.25 mm, 0.25 μm film thickness; DB-225, 30 m x 0.25 mm, 0.15 μm film thickness; J&W Scientific, Folsom, CA). Retention times of fatty acid methyl esters from tick salivary gland lipid samples and a standard mixture of fatty acid methyl esters on each column were compared. Samples from tick tissue and fatty acid methyl ester standards were also coinjected for authentication. Additional verification of the identity of salivary gland 20-carbon polyunsaturated fatty acids was obtained by gas-chromatography-mass spectrometry. Analyses

were conducted on a Hewlett-Packard 5790GC equipped with a 30 M x 0.25 mm, 0.2 μm film thickness Supelcowax 10 capillary column (Supelco, Bellefonte, PA). The GC was interfaced to a HP 5970 electron impact mass selective detector operated at 70 eV. Chromatographic conditions included a 45 s splitless injection, a 2-min hold period, and a 2°C/min temperature program from 150-250°C. Ultrapure He_2 was the carrier gas at 1 ml/min. Retention times and total ion mass spectra of FAMEs were compared with authentic standards from Sigma Chemical Company.

For routine analysis of fatty acid composition, samples were run on the DB-225 column temperature programed as follows: 120°C for 2 min, 10°C/min to 200°C, 5°C/min to 225°C, hold for 4 min. Samples were introduced using a split injector operating at a 1:20 split ratio and 250°C. Detection was with a flame ionization detector operating at 350°C.

Identification and Isolation of Lipid Classes

The lipid composition of the total lipid extract was determined by thin-layer chromatography on Analtech HPTLC uniplates. Plates were developed in one of three solvent systems: hexane-diethyl ether-acetic acid (90:10:1, 80:20:1, or 70:30:1), with a saturation pad added to insure uniform chamber saturation of solvent vapors. Before samples were separated, the thin-layer chromatography plates were predeveloped in the same solvent system

(90:10:1) and activated by heating to 100°C for 90 min. Separated lipids were visualized by charring with heat following immersion of the TLC plate in a solution of 3% cupric acetate in 15% phosphoric acid. The TLC plates were scanned with an Ultrascan XL laser densitometer (LKB, Bromma, Sweden) and peaks corresponding to location of lipids were compared to those of known lipid standards (Sigma 178-1 and 178-5 standards, containing monoglycerides, diglycerides, triglycerides, free fatty acids, cholesterol, and cholesterol esters).

Some lipid samples were separated on preparative TLC plates (Analtech) in hexane/diethyl ether/acetic acid (70:30:1) and assessed further for fatty acid composition by gas chromatography after separation and extraction from the silica gel. Plates were placed in an iodine chamber for visualization of lipids. Individual lipids were scraped from the plate into glass centrifuge tubes and neutral lipids (diglycerides, triglycerides, and free fatty acids) extracted with 3 ml diethyl ether. Polar lipids were extracted from the silica gel with 3 ml of chloroform/methanol/acetic acid/water (50:39:1:10) as described by Arvidson [22]. Two ml of 4 M NH_4OH were added to the extract, and lipids were recovered with chloroform. Fatty acid methyl esters were prepared as previously described before GC analysis.

Separation and Identification of Phospholipids and Associated Fatty Acids

Phospholipids collected from the previous chromatographic procedure were chromatographed further in chloroform/hexane/methanol/acetic acid/boric acid 40:30:20;10:1.8 (v/v/v/v/w) [23] on Whatman preparative TLC plates with preabsorbant zone. Individual phospholipids were visualized under iodine vapor and identified by comparing the chromatographic pattern to that of known phospholipid standards (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, lysophosphatidylcholine, lysophosphatidylethanolamine, cardiolipin, and sphingomyelin standards from Sigma), and each phospholipid was extracted as described by Arvidson [22]. Fatty acids from individual phospholipids were hydrolyzed, converted to methyl esters, and the fatty acid composition of each phospholipid determined by gas chromatography as described above.

Positional Distribution of Arachidonic Acid in Phospholipids

Purified salivary gland phospholipids containing arachidonic acid (phosphatidylcholine and phosphatidylethanolamine) were incubated with phospholipase A_2 (from Naja naja venom) to selectively hydrolyze fatty

acids from the sn-2 position and determine positional distribution of the fatty acid chains in salivary gland phospholipids [24]. Phosphatidylcholine and phosphatidylethanolamine obtained by TLC as described earlier were dried under nitrogen in a 7 ml scintillation vial and dissolved in 1 ml peroxide-free diethyl ether. Fifty μl of 0.1 M sodium borate buffer (pH 7.6) and 50 μl 0.1 M Tris-HCl buffer (pH 7.6) containing 0.4 M $CaCl_2$ were added with 200 units of phospholipase A_2 dissolved in 200 μl of 0.1 M Tris-HCl buffer. One sample of salivary gland PC and PE was co-incubated with a trace amount of L-3-Phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl to monitor the completeness of the reaction. Experiments were performed in airtight vials perfused with 100% N_2 before and after adding the enzyme, and samples were incubated at 37°C in a shaking water bath for 2 h. The ether was then evaporated under N_2 and lipids extracted by the methods of Bligh and Dyer [20]. Extracted samples were chromatographed on Whatman TLC plates as described for the separation of phospholipids. Lysophospholipids, free fatty acids, and unreacted phospholipids were visualized with iodine vapor and extracted from the silica gel by the method of Arvidson [22], and fatty acids in lipid fractions determined by gas chromatography as previously described. The radio-labeled samples were scraped into vials containing BioCount scintillation liquid (Research Products International, Mount Prospect, IL) and radioactivity

measured with a Beckman 3133T liquid scintillation counter (Fullerton, CA) to determine if all counts appeared in the free fatty acid fraction, indicating complete hydrolysis of the phospholipid into lysophospholipids and free fatty acids. Positional determination of fatty acids in phospholipids was assessed by comparing fatty acids in the phospholipid before the onset of the reaction to the location of fatty acids in unreacted phospholipid, lysophospholipid, and free fatty acid fractions upon completion of the reaction.

RESULTS

Fatty Acid Composition

Total fatty acids in lipid extracts of whole freshly dissected salivary glands from partially-fed lone star tick females as assessed by gas chromatography are shown in Figure 1. Oleic acid (18:1, n-9) was the most abundant fatty acid, comprising greater than 50% of the total (Table 1). Stearic (18:0) and linoleic acids (18:2, n-6) were 19% and 11% of the total respectively, followed by arachidonic acid (20:4, n-6) at 8%. Palmitic (16:0) and palmitoleic (16:1, n-9) acids were found in smaller amounts. The presence of arachidonic acid was confirmed by GC-MS (Fig. 2). Analysis of total lipid extracts by GC-MS also showed the presence of smaller amounts of eicosatrienoic acid (20:3, n-6) and eicosapentaenoic acid (20:5, n-6) (Fig. 3).

The retention times and mass spectra for these fatty acids were identical to authentic standards (data not shown).

Lipid Classes

The classes of lipids found in the salivary glands by thin-layer chromatography were phospholipids, diglycerides, sterols, free fatty acids, and triglycerides (Fig. 4). The identity of these lipids in the salivary gland were determined by comparison to authentic standards in three different solvent systems. The most abundant class of lipids, excluding sterols, was phospholipid, with over 87% of all fatty acids found in this fraction, as assessed by GC (Table 2). All arachidonic acid was associated with phospholipids in freshly dissected glands. Only small amounts of fatty acids were present in triglycerides and diglycerides and 0.5-14% were found as free fatty acids. The distribution of fatty acids in phospholipids was very similar to that in total lipid extracts from whole salivary glands.

Phospholipids

With the majority of fatty acids shown to be in phospholipids, they were examined further by TLC to identify subtypes. Those found included lysophosphatidylcholine, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, cardiolipin, and an unknown phospholipid which migrated

near the standard for sphingomyelin. The fatty acid composition in each phospholipid is shown in Table 3. Arachidonic acid was found only in phosphatidylcholine and phosphatidylethanolamine, while no arachidonic acid was associated with phosphatidylinositol. Phosphatidylcholine had 53% of all salivary gland fatty acids, but only 30% of the total arachidonic acid. While only 33% of the total fatty acids were found in phosphatidylethanolamine, 70% of all arachidonate was found in this salivary gland phospholipid (Table 4). In subsequent experiments, we have observed that the phospholipid location of arachidonic acid is quite variable and in some assays a higher percentage of 20:4 was found in PC than in PE (data not shown), suggesting that there is no preferential incorporation of arachidonic acid in one phospholipid over another. Reasons for this variability are unknown.

Lipids in Frozen Salivary Glands

The fatty acid composition and distribution in lipid extracts of stored and frozen glands were similar to that from fresh glands with two notable exceptions. A higher level of free fatty acids is seen in the frozen glands, including 7% of the total arachidonic acid (Table 5). The level of lysophosphatidylcholine was also elevated about 7% in the frozen glands (Table 6).

Positional Distribution of Arachidonic Acid in Salivary Gland Phospholipids

Because phospholipase A_2 is believed to be a major mechanism for phospholipase-induced generation of unesterified arachidonic acid, we were interested in assessing the position of esterified arachidonic acid in salivary gland phospholipids. Preliminary results indicate that a potent PLA_2 activity activated by micromolar Ca^{2+} is present in tick salivary glands (unreported data). Further, the equivalent amounts of non-arachidonic acid-containing lyso-PC and free arachidonic acid in frozen glands suggests a PLA_2-induced generation of unesterified arachidonic acid from PC in tick salivary glands.

The results of the positional distribution following enzymatic hydrolysis is summarized in Table 7. Saturated fatty acids were predominantly found in the sn-1 position of both PC and PE, while unsaturated fatty acids showed a preference for the sn-2 position, as assessed by analysis of lysophospholipids and free fatty acids after incubation of the phospholipids with snake venom PLA_2 for 2 h. Position 1 of PC contained about 80% of all 16:0 and 18:0, plus smaller amounts of 18:1, 18:2, and 20:4. In the PE fraction approximately 80% of 18:0 was found at sn-1, yet 16:0 was more evenly distributed between the sn-1 and sn-2 positions, with about 56% in position 1. Position 1 of PE also contained smaller percentages of 18:1, 18:2, and 20:4. No 16:1 was detected in the sn-1 position of either

phospholipid. Position 2 of both PC and PE were dominated by the unsaturated fatty acids 16:1, 18:1, 18:2, and 20:4. Approximately 75% of all arachidonic acid was detected in the sn-2 position of PC and PE.

DISCUSSION

Our knowledge of lipids in ticks is limited, with most of the studies in ticks focusing on lipids associated with the cuticle [25,26], eggs [27], and hemolymph [28,29]. Lipids are key biological molecules, serving as stores for metabolic energy, supplying constituents of signal transduction pathways, and providing fundamental components in the matrix of biological membranes. The majority of the lipids found in the salivary glands of A. americanum are phospholipids, although small amounts of diglycerides, triglycerides, sterols, and free fatty acids are also present. The high content of phospholipid is consistent with the highly membranous structure of the glands in partially-fed ticks [1] and the usual organelle location of phospholipids. The results suggests that stored lipids do not serve as major sources of high energy molecules in the salivary glands of partially-fed female ticks. Unsaturated fatty acids are associated with phospholipids in higher proportions than in the neutral lipids, while the saturated fatty acids are seen in higher percentages in the neutral lipids (triglycerides and diglycerides) in tick salivary glands. Stanley-Samuelson et al. [30] showed a

preferential incorporation of 20:4 over 18:0 in testicular phospholipids of the field cricket, Teleogryllus commodus. Twenty-carbon polyunsaturated fatty acids were also incorporated more readily than 18:1 and 16:0 into phospholipid fractions from the housefly, Musca domestica [31].

Phosphatidylcholine and phosphatidylethanolamine account for over 70% of total phospholipid in most of the species of insects studied [32,33]. PC is the major phospholipid class in tick salivary glands, paralleling the major phospholipids observed in Hymenoptera, Lepidoptera, and Orthoptera of the Class Insecta, while PE is the main phospholipid of Diptera [32,33]. The fatty acid composition of phospholipids in membranes can have a profound effect on the fluidity of the cell membrane [34], which can affect activity of membrane-bound enzymes and receptors, as well as permeability of the membrane to organic molecules. Generally, a more rigid plasma membrane has constituent phospholipids with a high proportion of saturated fatty acids. The fatty acids in the salivary glands of partially-fed females show a predominance of monounsaturated fatty acids (55% of the total), with smaller amounts of saturated (26%) and polyunsaturated (19%) fatty acids. Tick salivary glands have less palmitic acid (16:0), slightly more stearic acid (18:0), and zero linolenic acid (18:3) in comparison to whole body analyses of fatty acids in seven insect orders [35]. The

predominant fatty acid in tick salivary glands, as in most insects, is oleic acid (18:1). Twenty-carbon polyunsaturated fatty acids were not described in these earlier studies, possibly because of the sensitivity of assay methods then available.

This study has demonstrated a preferential incorporation of saturated fatty acids into the sn-1 position and unsaturated fatty acids into the sn-2 positions of phosphatidylcholine and phosphatidylethanolamine. This is consistent with distributions seen in mammalian and insect tissues studied to date. Eggs, larvae, and adults of the Mediterranean fruit fly, Ceratitis capitata, have a high degree of unsaturation in the 2-position of PC and PE that increases throughout the development of the insect [36]. Lambremont and Dial [37] showed similar distribution of unsaturated fatty acids in testicular PC and PE in the house cricket, Acheta domestica. No arachidonic acid was detected in either study, but an unknown 20-carbon fatty acid exclusively in the sn-2 position of PC and PE was reported by Lambremont and Dial [37].

The most striking result in our study is the relatively large amount of arachidonic acid in the salivary glands of female A. americanum. This is consistent with the high levels of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ in the saliva of partially-fed female lone star ticks [11]. The biosynthesis of eicosanoids is usually limited by the

availability of unesterified arachidonic acid serving as substrate for the cyclooxygenase and lipoxygenase enzymes [15]. The presence of arachidonate in phosphatidylcholine and phosphatidylethanolamine rather than other phospholipids is consistent with the distribution in the few arthropod cells and tissues studied to date. The quantity of arachidonate observed in the salivary glands (8% of total fatty acids) is greater than that found in the other arthropods. Arachidonic acid comprised 1.3% of all fatty acids in whole body lipid extracts of the American cockroach, Periplaneta americanum [38]. Wakayama et al. [39] detected 0.05% or less arachidonic acid in each lipid fraction from homogenates of the housefly, Musca domestica. Trace amounts of 20:4 were seen in all phospholipid fractions of Manduca sexta hemocytes [40].

Of further interest is the absence of detectable arachidonic acid in phosphatidylinositol in the salivary glands. The presence of 20:4 is a common characteristic of mammalian PI [41]. Phosphatidylinositol is a minor plasma membrane component, yet is important in signal transduction in cells. After PI is converted to diacylglycerol and inositol trisphosphate through the activation of phospholipase C, the diacylglycerol can activate protein kinase C [16] and inositol trisphosphate can cause the release of intracellular calcium [42]. The absence of 20:4 in PI supports the model of arachidonate release during cell activation, where arachidonic acid, which is

esterified to the sn-2 position of membrane-bound phospholipids, is released into the cytoplasm after activation of a phospholipase A_2. Release of arachidonic acid is thought to be the rate limiting step for eicosanoid production [43].

The higher levels of LPC and free arachidonic acid seen in frozen glands suggests that arachidonic acid may be released by the activation of a PLA_2 during the freezing, storage, and thawing of the salivary glands. This action of PLA_2 may be inhibited in the freshly dissected glands.

All work in this study was with the salivary glands of partially-fed female lone star ticks. Female lone star ticks feed for 8-14 days on a vertebrate host and reach a weight of 200-300 mg, and then enter a stage of fast feeding (12-48 hrs) during which a final weight of 600-900 mg is attained [44]. Distinct cytological changes occur in the salivary glands during these stages of feeding. A proliferation in mitochondria and intense interdigitation of plasma membranes are brought about, as well as an increase in cell size, mass, and protein content [1,45]. Salivary glands from ticks from later stages of feeding produce higher levels of inositol phosphates in response to a heat and trypsin sensitive factor from the tick brain (synganglion) than those from early feeding stages [46], suggesting that levels of phosphatidylinositol could change during tick feeding. Research focusing on changes in lipid composition, especially alterations in distribution of

arachidonate throughout the entire feeding process of the tick, will be valuable in further understanding the implications of the occurrence of arachidonate and eicosanoids in tick salivary glands.

Abbreviations used: BHT=butylated hydroxytoluene; CL=cardiolipin; EGTA= ethylene glycol bis-(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid; FAMEs= fatty acid methyl esters; GC-MS=gas chromatography-mass spectrometry; GHLF=gypsum hard layer with fluorescent indicator; HPTLC=high performance thin layer chromatography; LPC=lysophosphatidylcholine; MOPS=morpholinopropane sulfonic acid; PC=phosphatidylcholine; PE=phosphatidylethanolamine; PGE_2=prostaglandin E_2; $PGF_{2\alpha}$=prostaglandin $F_{2\alpha}$; PI=phosphatidylinositol; PIPES=piperazine N,N-bis-(2-ethane) sulfonic acid; PLA_2=phospholipase A_2.

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Table 1. Major fatty acids in freshly dissected salivary glands from partially fed *Amblyomma americanum* females.

Fatty Acid	Percent Composition	Micrograms per gland	Micrograms per mg protein
16:0	6.6 ± 0.8	1.6 ± 0.1	12.9 ± 2.7
16:1	3.4 ± 0.3	0.8 ± 0.1	6.9 ± 2.5
18:0	19.0 ± 2.9	4.5 ± 0.3	37.2 ± 6.5
18:1	51.7 ± 0.2	12.3 ± 1.1	103.5 ± 29.2
18:2	11.5 ± 1.5	2.7 ± 0.5	23.0 ± 8.1
20:4	7.8 ± 1.9	1.9 ± 0.6	16.4 ± 8.1

Mean ± S.D., n=3

Table 2. Percent fatty acid composition and percentage of each fatty acid occurring in the major lipid classes in tick salivary glands.

Fatty Acids	Phospholipids		Free Fatty Acids		Diglycerides		Triglycerides	
	% of lipid class	% total in this lipid class	% of lipid class	% total in this lipid class	% of lipid class	% total in this lipid class	% of lipid class	% total in this lipid class
16:0	5.4 ± 1.8	63.2 ± 6.1	26.5 ± 9.5	13.8 ± 1.5	31.5 ± 2.5	13.6 ± 3.3	17.5 ± 9.3	9.5 ± 3.2
16:1	2.4 ± 1.1	97.9 ± 3.7	ND	ND	ND	ND	1.0 ± 1.7	2.1 ± 3.4
18:0	18.2 ± 0.6	79.5 ± 6.8	39.9 ± 5.6	8.4 ± 3.3	33.0 ± 4.4	5.1 ± 0.6	31.4 ± 7.4	7.1 ± 3.4
18:1	51.2 ± 0.4	90.8 ± 2.8	32.3 ± 8.4	3.0 ± 2.2	32.5 ± 3.6	2.0 ± 0.3	44.9 ± 14.6	4.2 ± 2.3
18:2	12.4 ± 0.6	96.3 ± 1.1	1.3 ± 1.3	0.5 ± 0.4	3.0 ± 3.3	0.9 ± 1.0	5.3 ± 2.3	2.3 ± 1.7
20:4	10.3 ± 2.1	100	ND	ND	ND	ND	ND	ND

Percent composition ± S.D.; n = 3

Table 3. Percent fatty acid composition of the phospholipid classes in freshly dissected salivary glands.

Fatty Acids	LPC	PC	PI	PE	CL	UK
16:0	30.9 ± 6.6	10.8 ± 0.6	21.6 ± 2.6	5.5 ± 1.1	17.2 ± 13.9	28.0 ± 8.3
16:1	ND	3.8 ± 0.7	ND	1.7 ± 0.2	ND	ND
18:0	27.4 ± 7.1	18.7 ± 0.8	55.7 ± 10.5	23.2 ± 0.7	33.8 ± 14.9	42.3 ± 11.1
18:1	32.1 ± 8.2	53.0 ± 0.6	19.3 ± 4.6	47.8 ± 0.8	31.9 ± 17.2	29.5 ± 2.0
18:2	9.6 ± 9.0	10.9 ± 0.8	3.4 ± 3.9	10.6 ± 0.8	17.1 ± 16.4	ND
20:4	ND	3.0 ± 0.4	ND	11.4 ± 0.6	ND	ND

Means ± S.D., n = 3; UK = unknown, migrated near sphingomyelin; ND = not detected.

Table 4. Percentage of the total fatty acid and arachidonic acid present in phospholipid class.

Phospholipid	% Total Fatty Acids	% 20:4
LPC	1.7 ± 0.1	ND
PC	53.4 ± 1.0	29.6 ± 0.2
PI	2.2 ± 0.1	ND
PE	32.8 ± 1.7	70.4 ± 0.2
CL	7.9 ± 0.1	ND
UK	2.1 ± 0.4	ND

Means ± S.D., n = 3; UK = unknown, migrated near sphingomyelin; ND = not detected.

Table 5. Percentage of each fatty acid occurring in different major lipid classes in frozen salivary glands.

Fatty acid	Phospholipids	Free Fatty acids	Digylcerides	Triglycerides
16:0	79.9 ± 8.0	9.6 ± 3.7	5.7	8.6 ± 3.6
16:1	95.3 ± 4.2	4.7 ± 4.2	ND	ND
18:0	81.4 ± 6.1	8.5 ± 1.1	3.1	9.1 ± 6.2
18:1	91.2 ± 2.5	4.0 ± 0.7	0.7	4.5 ± 2.1
18:2	93.9 ± 1.2	6.1 ± 1.2	ND	ND
20:4	93.1 ± 1.8	6.9 ± 1.8	ND	ND

Mean ± S.D., n = 3; ND = not detected.

Table 6. Distribution of fatty acids among the major phospholipid fractions in frozen salivary glands of partially-fed Amblyomma americanum females.

Phospholipid	% Total Fatty Acids	% 20:4
LPC	7.7 ± 1.7	ND
PC	34.1 ± 4.1	41.3 ± 27.0
PI	8.7 ± 0.6	ND
PE	24.6 ± 3.2	58.7 ± 27.0
CL	13.8 ± 1.5	ND
UK	11.2 ± 3.6	ND

Mean percent composition ± S.D., n = 3; UK = unknown, migrated near sphingomyelin; ND = not detected.

Table 7. Distribution of fatty acids after incubating salivary gland phospholipids with snake venom phospholipase A_2.

Fatty acid	Phosphatidylcholine Position 1	Phosphatidylcholine Position 2	Phosphatidylethanolamine Position 1	Phosphatidylethanolamine Position 2
16:0	78.5 ± 10.3	21.5 ± 10.3	55.7 ± 11.6	44.3 ± 11.6
16:1	0	100	0	100
18:0	84.8 ± 7.0	15.2 ± 7.0	81.4 ± 16.5	18.6 ± 16.5
18:1	6.2 ± 1.3	93.8 ± 1.3	15.9 ± 8.7	84.1 ± 8.7
18:2	27.1 ± 6.1	72.9 ± 6.1	14.2 ± 11.5	85.8 ± 11.5
20:4	21.5 ± 10.8	78.5 ± 10.8	25.8 ± 16.2	74.2 ± 16.2

Mean percent composition ± S.D., n = 3 (PC) and 4 (PE).

Figure 1. Gas chromatogram of fatty acids present in a total lipid extract of salivary glands from partially-fed Amblyomma americanum females. Sample was separated on DB-225 column, operated as described in Materials and Methods.

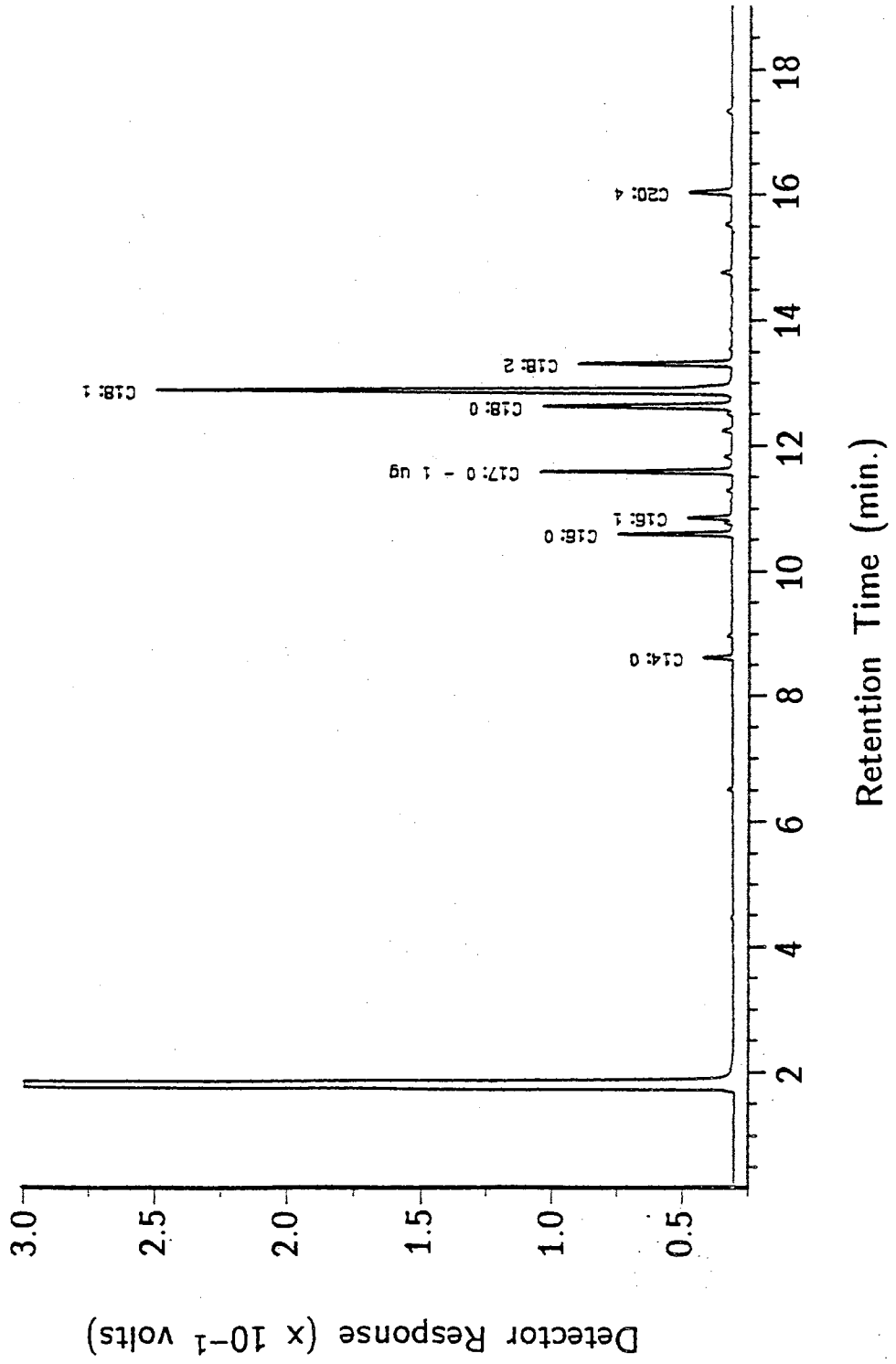

Detector Response (x 10⁻¹ volts)
3.0
2.5
2.0
1.5
1.0
0.5
2
4
6
8
10
12
14
16
18
Retention Time (min.)
C14: 0
C16: 1
C16: 0
C17: 0 - 1 ug
C18: 0
C18: 2
C18: 1
C20: 4

Figure 2. Mass spectra of (A) authentic arachidonic acid methyl ester standard and (B) a gas chromatography peak from a total lipid extract of tick salivary glands which eluted at identical retention time as authentic arachidonic acid methyl ester.

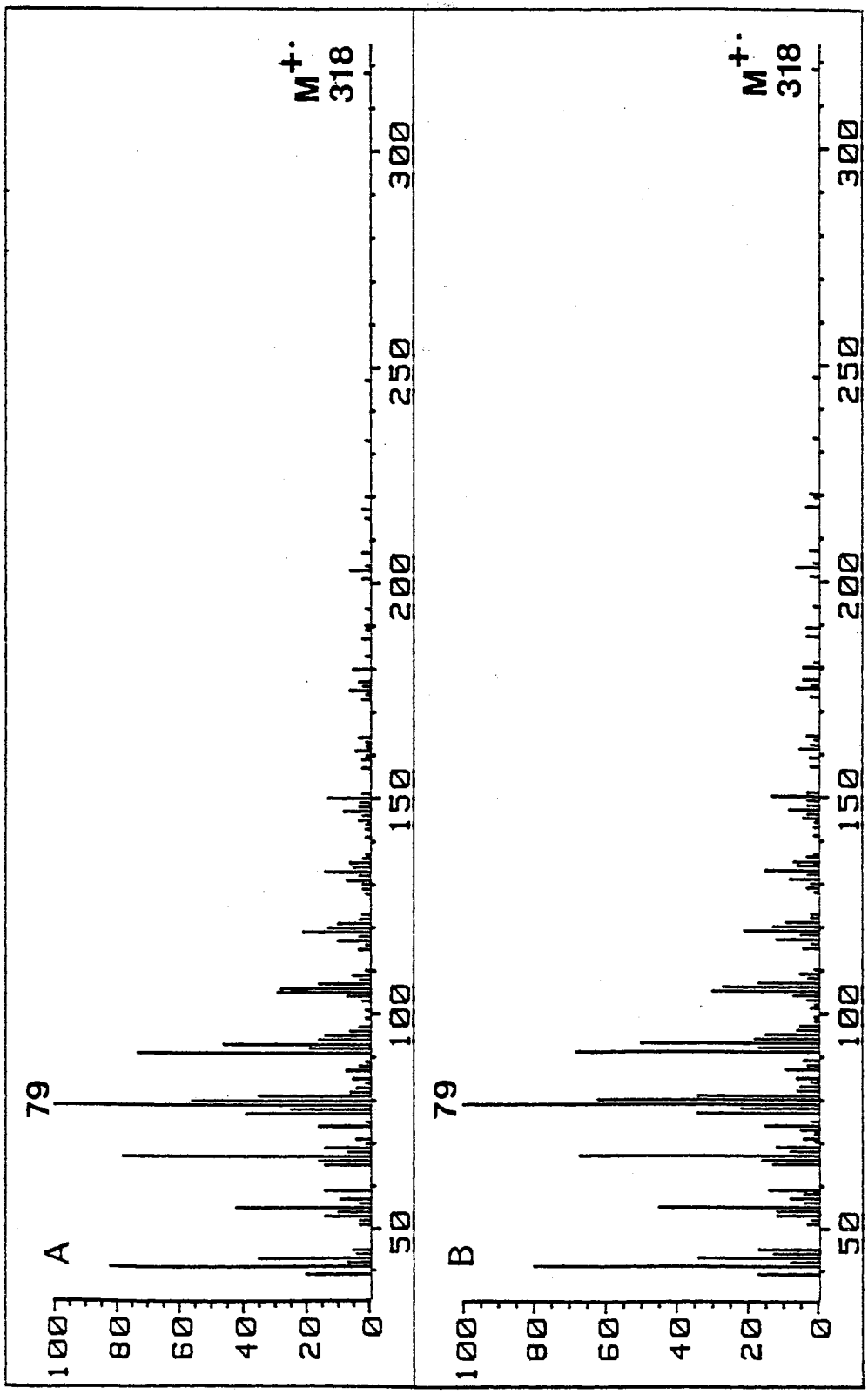

A
B
79
M+·
318
100
80
60
40
20
0
50
100
150
200
250
300
Percent of Base Peak
m/z

Figure 3. Total ion current gas chromatogram of fatty acids present in a total lipid extract of salivary glands from partially-fed Amblyomma americanum females. Sample was separated on Supelcowax 10 column. (A) total chromatogram (B) expansion of area containing twenty carbon polyunsaturated fatty acids.

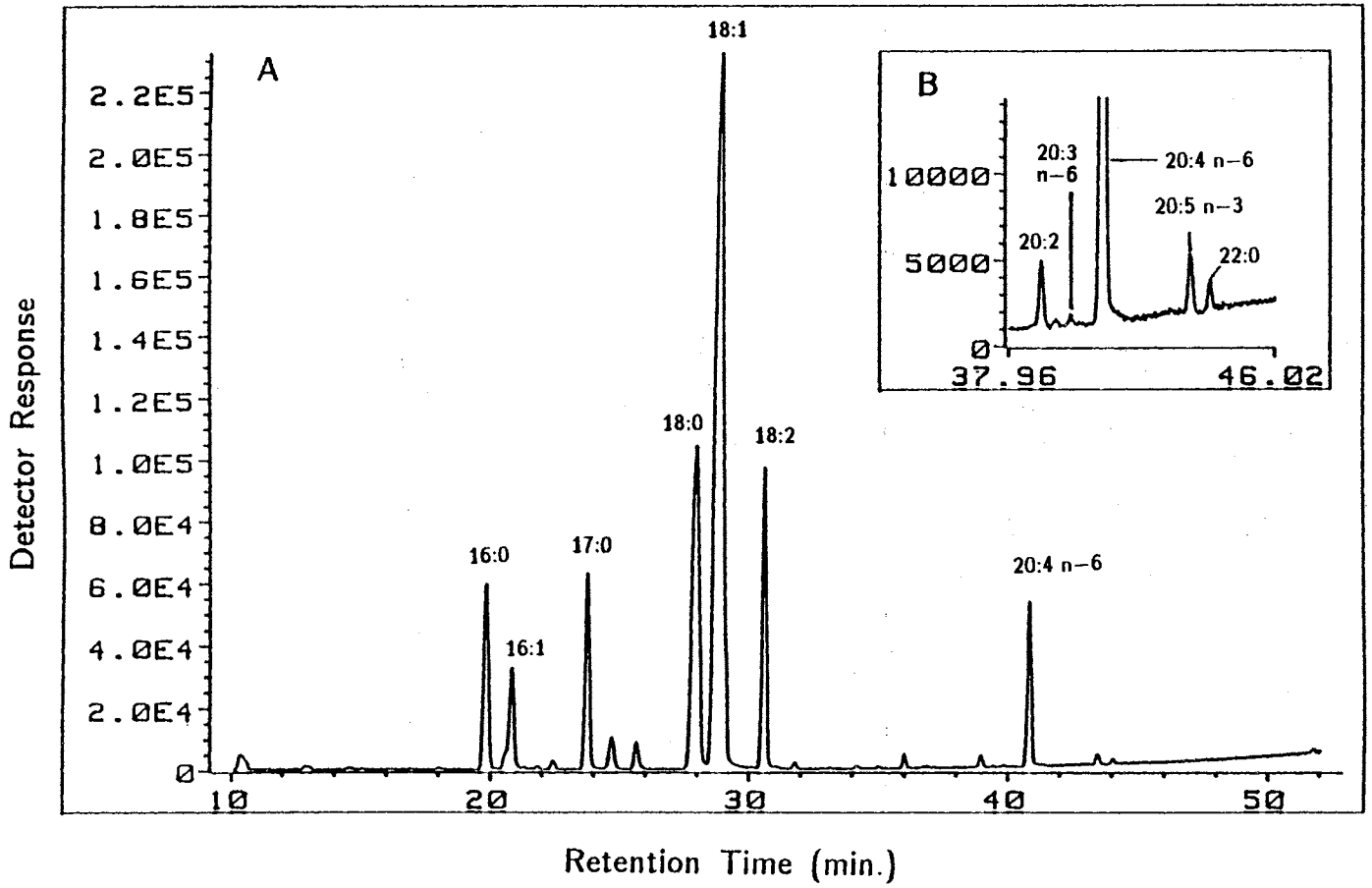
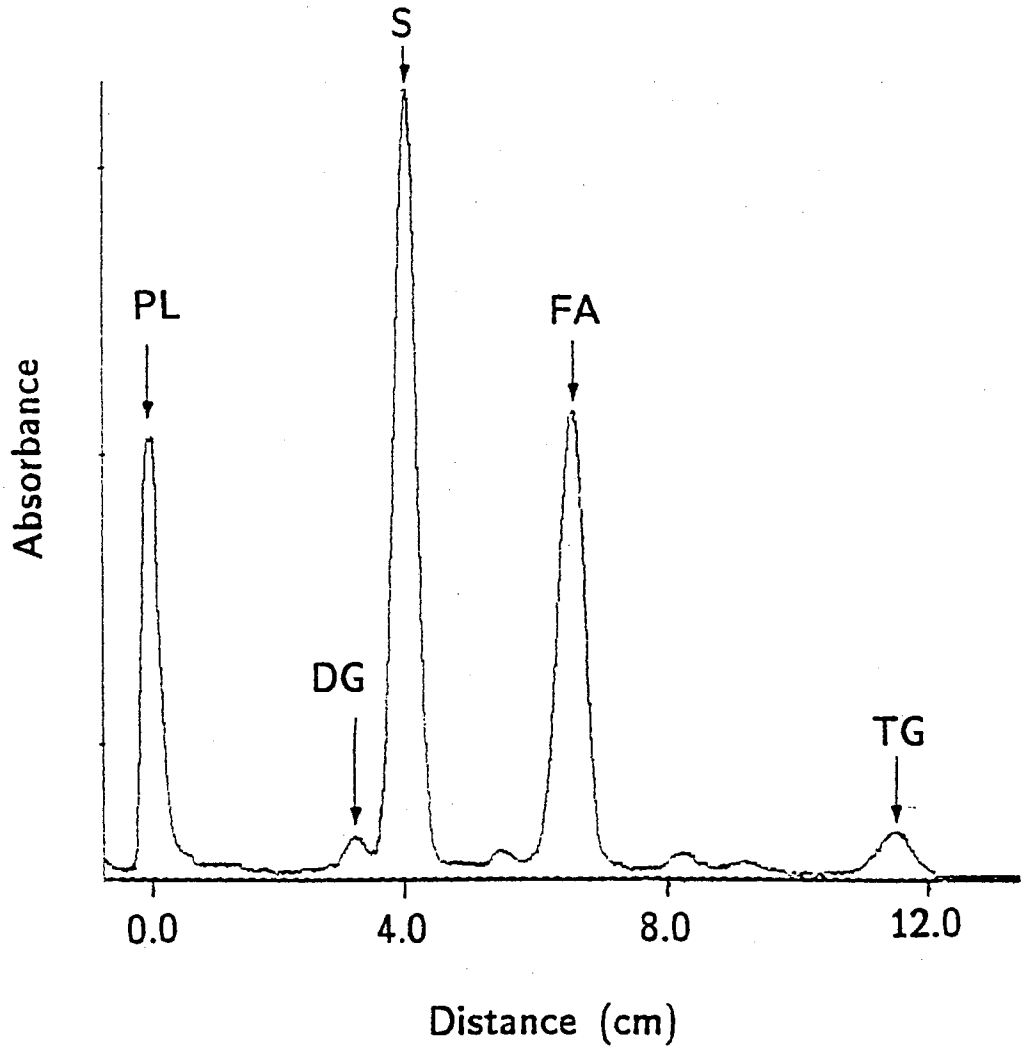

A
B
18:1
16:0
16:1
17:0
18:0
18:2
20:4 n-6
20:2
20:3 n-6
20:4 n-6
20:5 n-3
22:0
2.2E5
2.0E5
1.8E5
1.6E5
1.4E5
1.2E5
1.0E5
8.0E4
6.0E4
4.0E4
2.0E4
0
10000
5000
0
37.96
46.02
10
20
30
40
50
Detector Response
Retention Time (min.)

Figure 4. Laser densitometric scan of tick salivary gland lipids separated on silica gel high performance thin-layer chromatography plate. The solvent system used was hexane/diethyl ether/acetic acid (70:30:1). PL = phospholipids; DG = diglycerides; S = sterols; FA = free fatty acids; TG = triglycerides.


S
PL
FA
DG
TG
Absorbance
0.0
4.0
8.0
12.0
Distance (cm)

PART II

CHANGES IN LIPIDS FROM THE SALIVARY GLANDS OF THE LONE STAR TICK, *AMBLYOMMA AMERICANUM*, DURING FEEDING

ABSTRACT

The lipid composition of salivary glands from male and female lone star ticks, Amblyomma americanum, was investigated at progressive stages of tick feeding. The amounts of fatty acids from both phospholipid and neutral lipid fractions increased dramatically during the initial stage of feeding, and peaked in partially-fed females weighing 100-250 mg. Percent compositions of myristic (14:0) and palmitic acid (16:0) decreased, but stearic (18:0), oleic (18:1), linoleic (18:2), and arachidonic acid (20:4) increased during tick feeding. Arachidonic acid, the precursor to eicosanoids including the 2-series of prostaglandins, increased from 1.3% of all fatty acids in salivary glands from unfed female ticks to 8.2% in salivary glands from fully engorged female ticks. Arachidonic acid was found in the triglyceride fraction of unfed and fed virgin females, but only in phosphatidylcholine and phosphatidylethanolamine from salivary glands of other fed female ticks. Comparisons between fed and unfed male ticks and fed/virgin, fed/mated, and unfed females demonstrate that feeding is necessary for accumulation of arachidonic acid in salivary gland phospholipids.

INTRODUCTION

Ticks are widely recognized as economically important pests and efficient vectors of pathogens to man, domestic animals and wildlife. Ticks surpass all other arthropods in the variety of pathogens they transmit to hosts, which include protozoa, rickettsiae, bacteria, viruses, fungi, and filaria (Sonenshine, 1991). A wide distribution and abundance of species, their longevity and high reproduction rates, and extended periods of feeding are factors which enable ticks to be prolific parasites and suitable vectors of these agents of disease (Friedhoff, 1990).

The great success exhibited by ticks as vectors is also facilitated by pharmacologically active compounds found in tick saliva. An anticoagulant secreted into the feeding lesion interrupts the coagulation cascade of the host (Waxman et al., 1990; Gordon and Allen, 1991) and an apyrase activity in the saliva of some species counteracts hemostasis by preventing platelet aggregation (Ribeiro et al., 1985). Factors in the saliva of Ixodes dammini can destroy bradykinin and anaphylatoxins, and can prevent expression of complement-mediated inflammation (Ribeiro, 1987). Additionally, the natural immunity mounted by the host against tick feeding is countered by components in tick saliva. T-cell activation is prevented by salivary

components of I. dammini (Ribeiro et al., 1985), and salivary gland extracts of Dermacentor andersoni suppresses T-lymphocyte cytokine secretion (Ramachandra and Wikel, 1992).

Prostaglandins have been identified in the saliva of several species of ticks (Higgs et al., 1976; Dickenson et al., 1976; Ribeiro et al., 1985; Ribeiro et al., 1992) and are postulated to aid the tick in overcoming hemostasis, inflammatory responses, and host immunity. Prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) can prevent platelet aggregation and cause vasodilation, which maintains an ample supply of blood flowing to the tick mouthparts (Ribeiro, 1987). PGE_2 and PGI_2 also inhibit mast cell degranulation, which helps to minimize release of inflammatory mediators (Bach, 1982; Davies et al., 1984). Secretion of the cytokines interleukin -1 and -2 is suppressed by PGE_2 and PGI_2 causing inhibition of T-cell clonal expansion (Hecker et al., 1988).

The precursor to the 2-series of prostaglandins, arachidonic acid (20:4, n-6), was recently identified at relatively high levels (8% of total fatty acids) in the salivary glands of partially-fed Amblyomma americanum females (Shipley et al., 1993). Arachidonic acid is liberated from membrane lipids by a phospholipase A_2 (PLA_2) in most cells, where it is rapidly converted to prostaglandins and other eicosanoids by prostaglandin synthetase (Smith and Marnett, 1991).

The salivary glands of ixodid ticks play a vital role in the osmotic and ionic regulation of the parasite (Kaufman and Sauer, 1982) in addition to providing a site for development of pathogenic microorganisms. Great changes in the physical and functional properties occur in tick salivary glands throughout the process of feeding. The cells of the granular alveoli (types II and III) are largely undeveloped in the unfed female, but hypertrophy during feeding (Fawcett et al., 1986) as the cells differentiate to a transport function. While males feed little after attachment to the host (Sauer and Essenberg, 1984), the female lone star tick feeds at a slow rate for 8-14 days, increasing from an unfed weight of 4 mg to a weight of about 200-300 mg. The tick then enters a state of rapid feeding and attains a weight of 600-900 mg in 12-48 hrs (Sauer et al., 1979). Mating is necessary to initiate these feeding-induced changes in the salivary glands.

The apparent importance of prostaglandins in the successful parasitization of vertebrate hosts by ticks and the significant changes observed in salivary glands during feeding led to the investigation of the occurrence and distribution of arachidonic acid and other fatty acids in the salivary glands of adult lone star ticks from various stages of feeding. Here we compare the salivary gland fatty acid composition of unfed female ticks to partially-engorged, fully-engorged, and virgin female ticks to

determine the effects of feeding and mating on arachidonic acid and other lipid molecules in the cells of tick salivary glands.

MATERIALS AND METHODS

Materials

All lipid and fatty acid methyl ester standards, boron trifluoride and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co., St. Louis, MO. Preparative silica gel 60 A thin-layer chromatography plates were acquired from Whatman, Maidstone, England (LK6F plates: Linear-K pre-absorbent zone; 20 x 20 cm, 250 μm thickness), and Analtech, Newark, DE. (silica gel GF uniplates; 20 x 20 cm, 500 μm thickness). Silicic acid (Bio-Sil A; 100-200 mesh) was purchased from Bio-Rad Laboratories, Richmond, CA. Diethyl ether was obtained from EM Science, Gibbstown, NJ. All other solvents were reagent grade from Fisher Scientific, Pittsburgh, PA, and were redistilled in glass before use.

Collection of tick salivary glands

Lone star ticks were laboratory reared according to the methods of Patrick and Hair (1975). Larvae were hatched from eggs in a humidity chamber (27-28°C; 90-100% relative humidity) and fed on rabbits until repletion. After molting off the host, nymphs were placed on sheep and

fed for 3-5 days before they detached and molted. All adult ticks were placed within surgical stockinettes affixed to the skin of sheep and were allowed to feed for various lengths of time prior to removal. Some ticks were allowed to feed to engorgement and drop from the host before use in experiments. Male ticks were placed on the host with females and were collected after mating and feeding. Virgin female ticks were obtained by placing female adult ticks in a stockinette in the absence of males and allowing to feed for 12-13 days. Unfed adult ticks were maintained at 27-28°C and 90-100% relative humidity before infestation of the host or use in experiments. Fed female ticks were weighed on an analytical balance and placed within a group based on tick weight: 4-20, 20-50, 50-100, 100-250, and >250 mg. Salivary glands were removed at 4°C in 0.1 M morpholinopropane sulfonic acid buffer (MOPS, pH 6.8) with 20 mM ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Salivary glands were pooled and weighed on an analytical balance to record salivary gland mass. The number of ticks used in each experiment differed according to the weight of the tick. Unfed female ticks required 30-40 ticks per experiment while 10-15 rapid feeding ticks (>250 mg) was sufficient to provide an adequate amount of salivary gland tissue for lipid analysis.

Extraction of total lipid

Salivary glands were suspended in 800 μl 0.1M MOPS, pH 6.8, containing 20mM EGTA, or in 1 ml chloroform to insure inhibition of phospholipase activity, and macerated in a glass homogenizer. Methanol and water were added and the phases separated by the method of Bligh and Dyer (1959). Extracted lipids were stored at -20°C in chloroform with 0.05% BHT added to prevent oxidation of lipids.

Separation of lipid classes

Total lipid samples were fractionated by thin-layer chromatography (TLC) on silica gel GF uniplates. Plates were pre-washed in hexane-diethyl ether-acetic acid (70:30:1) and then developed in the same solvent system. Following visualization with iodine vapor, silica gel containing lipid samples were scraped from the plate and neutral lipids were extracted with 3 ml diethyl ether. Polar lipids (phospholipids) were extracted from the silica gel in 3 ml of chloroform-methanol-acetic acid-water (50:39:1:10; Arvidson, 1967). Two ml of 4M NH_4OH were added to the extract, and lipids were recovered with chloroform and stored at -20°C in 0.05% BHT in chloroform.

The isolated phospholipid fraction was re-chromatographed in chloroform-hexane-methanol-acetic acid-boric acid 40:30:20;10:1.8 (v/v/v/v/w) on Silica Gel 60 A Whatman preparative TLC plates with preabsorbant zone. Individual phospholipids were identified and extracted from the plates as previously described.

Preparation and analysis of fatty acid methyl esters

Fatty acids from individual neutral lipids, phospholipids, and total (unfractionated) lipid extracts were hydrolyzed and converted to methyl esters for analysis by gas chromatography (GC). Lipid extracts were placed in glass vials and dried under a stream of nitrogen gas, with 50 μl of a 1 mg/ml solution of heptadecanoic acid methyl ester (17:0) added as an internal standard. Lipids were hydrolyzed with 1 ml 5% KOH in methanol at 60°C for 90 minutes and fatty acid methyl esters (FAMEs) were prepared by incubating with 2 ml boron trifluoride (14% in methanol) at 60°C for 30 minutes (Ryan et al. 1982). Fatty acid methyl esters were extracted three times with 2 ml chloroform, and dehydrated with $MgSO_4$. Samples were evaporated under nitrogen, resuspended in 200 μl hexane, and added to a pipette containing 100-200 mesh silicic acid (Bio-Sil A) that had been activated at 100°C for 90 min. Hydrocarbons were eluted from the pipette columns with 3 ml ether, and then FAMEs were eluted from the pipette with 6 ml of 5% diethyl ether in hexane.

Fatty acids were assessed by gas chromatography (Hewlett-Packard 5890 equipped with a flame ionization detector) on a fused silica column (DB-225, 30 m x 0.15 mm, 0.15 μm film thickness; J&W Scientific, Folsom, CA). Retention times of FAMEs from tick salivary gland lipid samples and a standard mixture of FAMEs were compared. The

chromatographic conditions used follow that of Shipley et al. (1993). Verification of purified tick salivary gland arachidonic acid from triglycerides and free fatty acids of unfed ticks was assessed by gas chromatography-mass spectrometry (GC-MS), using a Hewlett-Packard 5790GC equipped with a 30 M x 0.25 mm, 0.2 μm film thickness Supelcowax 10 capillary column (Supelco, Bellefonte, PA). The GC was interfaced to a HP 5970 electron impact mass selective detector operated at 70 eV. Chromatographic conditions included a 45 sec splitless injection, a 2-min hold period, and a 2°C/min temperature program from 150-250°C. Ultrapure helium was the carrier gas at 1 ml/min. Retention times and total ion mass spectra of FAMEs were compared with authentic standards from Sigma Chemical Co.

RESULTS

Salivary gland mass during tick feeding

The salivary glands of female lone star ticks greatly increased in mass after the onset of tick feeding (Table I). The salivary glands enlarged by over 200% after the first 4-7 days of feeding, and continued to increase in size until females attained a weight of 100-250 mg. Salivary gland mass did not increase as the tick entered the stage of rapid feeding. Salivary glands of virgin females were similar in mass to 4-20 mg fed female ticks. Male tick salivary glands increased by 60% after 11-14 days

of intermittent feeding (Table I).

Fatty acids in salivary gland phospholipids and neutral lipids

The lipid classes identified in the salivary glands during all stages of female tick feeding were phospholipids (PL), diglycerides (DG), free fatty acids (FA), triglycerides (TG), and sterols (S). Additionally, a sterol ester (SE) fraction was identified by co-migration with authentic standards in unfed, virgin, and replete female ticks.

The phospholipid fraction was by far the most abundant lipid class, contributing the majority of fatty acids in tick salivary glands. Total fatty acids in phospholipids rose sharply after tick attachment and feeding, and steadily increased before reaching a plateau (Fig. 1). Fatty acids of neutral lipids generally increased in a similar manner during feeding, but with lower amounts. Free fatty acids increased after the onset of feeding and then remained fairly constant (Fig 2), while fatty acids associated with diglycerides rose slowly throughout the feeding process (Fig 3). Triglycerides had higher amounts of associated fatty acids than any other neutral lipid in all phases of tick feeding, increasing 3-fold soon after feeding began (Fig. 4). Sterol esters were detected in small amounts in the salivary glands of unfed, replete, and virgin female ticks, but were not detected in any other

feeding stage (Fig 5).

The changes in fatty acids of individual phospholipids during feeding is summarized in Figures 6-10. Phospholipids detected in salivary glands from all stages of tick feeding were phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lysophosphatidylcholine (LPC), and cardiolipin (CL). An unknown polar lipid, detected in salivary glands from partially-fed female ticks by Shipley et al., (1993), was not investigated in this study as it appears infrequently in sufficient quantity to visualize on TLC plates.

Phosphatidylcholine was the most abundant salivary gland phospholipid in all feeding stages as determined by the quantity of fatty acids associated with this phospholipid class (Fig. 6). The amount of fatty acids in PC rose sharply at the onset of feeding with a high of 3.60 μg/gland in the 100-250 mg tick feeding stage, and then declined as the tick attained repletion. Changes in amounts of fatty acids in PE paralleled those seen in PC, but the quantities were lower in all stages of tick feeding (Fig 7). The quantity of fatty acids in PI increased during feeding, with a high of 0.69 μg/gland in the 100-250 mg feeding stage (Fig. 8). The 100-250 mg tick weight range also contained the largest amounts of salivary gland LPC fatty acids (Fig 9) while the majority of fatty acids associated with cardiolipin were found in replete ticks (Fig 10). Virgin females had a phospholipid fatty acid

content greater than that found in salivary glands of unfed female ticks, but less than that found in feeding ticks of the 4-20 mg range.

Arachidonic and linoleic acid composition as a function of tick weight

Arachidonic acid was present in total lipid extracts from all stages of feeding ticks (Fig. 11). Arachidonic acid comprised 1.3% of fatty acids in salivary glands of unfed females and increased to 5% in the 4-20 mg stage of feeding. In the 20-50 mg stage arachidonic acid increased to 7.6% of total fatty acids, and remained near this percentage throughout the duration of feeding. Amounts of arachidonic acid in each gland, also included in Figure 11, increased 40-fold during feeding, reaching a high of 1.64 μg/gland in rapid feeding ticks (>250 mg). The amount of arachidonic acid per gland in replete ticks, measured within 24 hr after dropping from the host, showed a decrease although the percent composition remained high. Virgin females had a high percentage of arachidonic acid (7.6%) but had an amount three times less than ticks weighing >250 mg.

In most feeding stages arachidonic acid was associated entirely with PC and PE (Table II). PC contained over three times more arachidonic acid than PE in some stages of tick feeding. Unfed and virgin female ticks contained a small amount of arachidonic acid in PI, while none was

detected in PI from salivary glands of other feeding ticks. Additionally, a relatively large amount of arachidonic acid was found in the triglycerides of unfed and virgin female ticks, and salivary glands from unfed females contained some unesterified arachidonic acid. This free and triglyceride-bound arachidonic acid was verified as authentic by GC-MS (data not shown). About 67% of the total amount of arachidonic acid in unfed female salivary glands was found in the triglyceride fraction, compared to 23% for virgin females. A smaller percentage (13%) was found as free arachidonic acid in unfed females. No arachidonic acid was found in neutral lipids from other stages of tick feeding. Linoleic acid (18:2) was present in all feeding stages in higher quantities than arachidonic acid (Fig. 12). Linoleic acid rose from 5% in unfed ticks to 16% of the total from rapid feeding ticks.

Changes in fatty acid composition during feeding

The changes in percent composition of all detected salivary gland fatty acids during tick feeding are shown in Figure 13. Myristic (14:0) and palmitic acid (16:0) decreased in percent composition during feeding (Fig. 13A). The percent decrease in 16:0 is dramatic between unfed females and 20-50 mg female ticks, dropping from 22% to 8% of total fatty acids. Stearic acid (18:0) showed a small overall increase in percent composition during feeding. The unsaturated fatty acid composition in the salivary

glands increased during the intake of the bloodmeal. The most abundant fatty acid in all stages of tick feeding was oleic acid (18:1), comprising as much as 53% of the total fatty acids (Fig. 13B). Palmitoleic acid (16:1) composition remained relatively constant during feeding. Arachidonic and linoleic acid are included in Figure 13B for comparative purposes.

All fatty acids show increases in amounts per gland as the tick takes a bloodmeal. By far the largest rise is seen by arachidonic acid which shows a 40-fold increase (Table III). Linoleic acid increased by 20-fold during the feeding process of the tick, while the remaining detected fatty acids had smaller increases.

Arachidonic acid in fed and unfed male ticks

To investigate how feeding, mating, and gender affects salivary gland fatty acids, percent compositions of arachidonic acid in total lipid extracts of salivary glands from fed and unfed males were compared with unfed, fed/virgin, and fed/mated females (Fig. 14). There was no difference in arachidonic acid composition between fed males (2.5% ± 0.4) and unfed males (1.4% ± 0.1; $P>0.05$). In actual amounts, fed males had a 3-fold increase compared with unfed male ticks. Percent compositions of arachidonic acid in unfed male and unfed female (1.3% ± 1.0) tick salivary glands were similar (Fig. 14), yet the unfed females had 5-fold more arachidonic acid. Arachidonic acid

percent composition increased more dramatically in females during feeding as compared to males, which take a relatively small bloodmeal. Virgin females had a similar percent composition ($P>0.05$) of arachidonic acid as mated female ticks that fed for a similar time. Fed females (virgin or mated) had a greater percentage arachidonic acid content ($P<0.01$) than unfed females (Fig. 14).

DISCUSSION

The increase in salivary gland mass and fatty acids associated with phospholipids are indicative of the dramatic changes that occur in the salivary glands as the tick ingests a bloodmeal. Salivary glands in ixodid ticks consist of a pair of clustered alveoli that surround a central salivary duct. The size and mass of individual granular alveoli (types II and III) greatly increase, although the numbers of alveoli do not change (Fawcett et al., 1986). The cells increase in mitochondria and acquire thin processes that form a basal labyrinth that is thought to function as a fluid transporting epithelium (Fawcett et al., 1981; Gill and Walker, 1987). An extensive network of swollen rough endoplasmic reticulum is formed, and protein content is greatly increased during feeding (McSwain et al., 1982). These changes appear to be stimulated by a hemolymph-borne factor (Coons and Kaufman, 1988) which may involve juvenoid and ecdysteroid molecules (Shelby et al., 1989).

The most significant changes in gland mass and fatty acid composition occur when ticks are in the 8-14 day "slow feeding" stage and for this reason most of the tick weight ranges chosen in this study were below 250 mg. Adult female A. americanum are not consistent in their feeding habits, so it is difficult to accurately predict the amount of blood consumed on a time basis after placing ticks on the host (Sauer and Hair, 1972; Hume et al., 1985).

The salivary glands of mated females lose competence to secrete and degenerate following repletion. A humoral tick salivary gland degeneration factor (TSGDF), thought to be 20-hydroxy-ecdysone, is responsible for the alveolar autolysis that occurs as resources of the bloodmeal become mobilized for egg production (Kaufman, 1986). Glands of replete female ticks had reduced mass and appeared more diffuse compared to feeding ticks, yet phospholipid content remained high in replete females. Salivary glands from ticks in the rapid feeding stage (>250 mg) weighed less than those from the 100-250 mg stage, perhaps because of continuing secretion or because ticks nearing repletion had undergone some salivary gland autolysis.

Most of the salivary gland fatty acids were associated with the phospholipid (PL) class, which is indicative of the highly membranous structure of the glands. The most abundant neutral lipid in tick salivary glands observed in this study are the triglycerides (TG), which typically function as the major storage form for fatty acids in most

insects (Stanley-Samuelson et al., 1988). Triglycerides tend to be the major source of metabolic energy in insects which undergo prolonged periods of metabolic activity without feeding and during non-feeding stages of development (Downer, 1985). The ratio of PL/TG is about 18:1 in rapid feeders, yet is nearly 1:1 in unfed females.

The most abundant phospholipid molecules in all feeding stages were PC and PE, resembling that seen in most orders of insects (Downer, 1985). In the few phospholipid studies in ticks (Hajjar, 1972; Maroun, 1972; Kamal and Kamel, 1977), no further fractionation into composite phospholipid classes were performed. The general increase in PI during feeding corroborates the work of McSwain et al. (1989), in which salivary glands from rapid feeding ticks produced higher levels of inositol phosphates than ticks from early stages of feeding in response to a heat and trypsin sensitive factor from the tick brain (synganglion). Phosphatidylinositol is a minor plasma membrane component, yet is important in transmembrane signalling in many cells.

Of considerable interest is the distribution and increase of arachidonic acid during the feeding process. In previous studies on partially-fed female ticks (below 200 mg), arachidonic acid comprised 7.8% of a total lipid extract from lone star tick salivary glands and was found only in PC and PE (Shipley et al., 1993). The percent composition of arachidonic acid at the equivalent feeding

stage in this study (100-250 mg) was similar at 7.25%. Arachidonic acid was found only in PC (75.5% of total) and PE (24.5%) in the 100-250 mg stage, which differs from results obtained in our previous study which showed 70% of all arachidonic acid associated with PE and 30% with PC (Shipley et al., 1993). Therefore, there appears to be some variation in the distribution of arachidonic acid between salivary gland PC and PE.

The pattern of salivary gland arachidonic acid increase raises questions regarding the source of the fatty acid. Arachidonic acid could be acquired in the bloodmeal and stored primarily in phospholipids or synthesized de novo or from precursors such as linoleic acid. Metabolic conversion of linoleic to arachidonic acid and other twenty-carbon polyunsaturated fatty acids readily occurs in most insects (Stanley-Samuelson et al., 1986, Jurenka et al., 1987, Dadd et al., 1987) and the elongation/desaturation pathways of vertebrates are typically employed. An exception is the mosquito Culex pipiens which lacks the ability to convert linoleic acid to arachidonic acid (Dadd, 1983). In recent experiments, the lone star tick was unable to synthesize arachidonic acid from linoleic acid or any other precursors (Bowman et al., 1993), which suggests that the tick must obtain arachidonic acid from the bloodmeal like the mosquito. Thus, the increase in percent composition and amounts of arachidonic acid in the salivary glands during feeding represents an

uptake and sequestration of the fatty acid, primarily in the phospholipids.

The biosynthesis of linoleic acid was once thought not to occur in any animal system, yet this capability has been shown in several insect species (Blomquist et al., 1982; de Renobales et al., 1986). The tick is similar to most animals as it lacks the ability to synthesize linoleic acid from oleic acid (Bowman et al., 1993), so the increase of linoleic acid in this study represents an accumulation from the bloodmeal as seen with arachidonic acid.

Additional studies have demonstrated that arachidonic acid is preferentially incorporated into the salivary glands of A. americanum when compared to other tissues. A higher percent composition is seen in salivary glands than ovaries, Malphigian tubules, and hemolymph (Bowman et al., 1993). This evidence supports the hypothesis that arachidonic acid is utilized in the tick primarily for biosynthesis of prostaglandins, which are secreted into the feeding lesion to counteract the host's natural defenses or to halt the coagulation of blood (Ribeiro, 1987). The increase in arachidonic acid as feeding begins coincides with the time that the pro-feeding characteristics of prostaglandins are needed by the tick. Arachidonic acid constitutes a small percentage of fatty acids from unfed adult ticks, and its occurrence perhaps is the remnant of larval and nymphal feedings.

The distribution of arachidonic acid in the tick

salivary glands is consistent with the typical location of the fatty acid for eicosanoid biosynthesis. The primary source of free arachidonic acid is thought to be through the action of phospholipase A_2, which releases arachidonic acid from the 2-position of membrane phospholipids (Lapetina, 1990). Arachidonic acid is primarily esterified to the sn-2 position (75%) of salivary gland phospholipids (Shipley et al., 1993). Phospholipase A_2 activity, measured by release of labelled arachidonate from phospholipids, is present in the salivary glands of A. americanum (Sauer et al., 1993), and phosphatidylcholine is the preferred substrate of the enzyme.

The salivary glands of both unfed and virgin females contained high amounts of arachidonic acid in the triglyceride fraction. Long-chain polyunsaturated fatty acids are usually found in phospholipids in most animals, including insects (Stanley-Samuelson et al., 1988), yet small amounts occur in TG fractions of some insect species including the waxmoth, American cockroach, and housefly (Stanley-Samuelson and Dadd, 1983; Wakayama et al., 1985). The triglyceride fraction of unfed Anopheles stephensi mosquitos had 0.4% arachidonic acid, while TGs from blood-fed mosquitos had 0.2% from whole body extracts (Stanley-Samuelson and Dadd, 1983), a pattern similar to the tick salivary glands. When specific tissues are analyzed, the percentage is often increased. Arachidonic acid comprised 12.6% of the TG fraction from larval head extracts of the

tobacco hornworm, Manduca sexta (Ogg and Stanley-Samuelson, 1992). In studies with the field cricket, Teleogryllus commodus, 30% of the radioactivity taken up by testes from [^{3}H]-20:4 was incorporated into the triglyceride fraction, and 68% of radioactivity was incorporated in TG of the male fat body (Stanley-Samuelson et al., 1986). The large proportion of arachidonic acid in triglycerides of unfed female tick salivary glands may act as a temporary reserve to subsequently be utilized in the synthesis of membrane phospholipids and for eicosanoid biosynthesis upon initiation of tick feeding. Triglycerides have been shown to function as a store for arachidonic acid in other tissues including human endometrial cells (Bonney et al., 1991) and rabbit renal medulla cells (Liston and Nasjletti, 1987), and can serve as a source of arachidonic acid for prostaglandin synthesis in the thyroid (Haye et al., 1974).

Other salivary gland fatty acids changed during tick feeding, especially in the early stages. Fatty acid compositions of phospholipids can affect the fluidity of the plasma membrane (Downer, 1985), which may in turn alter the permeability of the membrane and affect activity of membrane-bound receptors and enzymes. Tick salivary glands showed a general increase in unsaturated fatty acids and a decrease in saturated fatty acids, leading to a more fluid membrane which may facilitate secretion and salivary gland differentiation during feeding. Mating is necessary for female ticks to acquire a large blood meal (McSwain et al.,

1982) and fed/virgin female ticks in this study did not attain a weight beyond 40 mg. Virgin female ticks, however, had a percent arachidonic acid content similar to fed/mated females. Significant differences of percent composition of arachidonic acid between unfed and fed females, with no differences between fed/virgin and fed/mated ticks suggest that attachment and feeding is the trigger that initiates incorporation of arachidonic acid into tick salivary glands, as opposed to mating.

The fatty acid composition of a particular organism or tissue may be viewed as a window into a complex, ongoing network of dynamic processes (Stanley-Samuelson et al., 1988). Alterations of profiles in response to developmental parameters or environmental conditions suggest that fatty acid compositions have physiological relevance. The changes seen in the salivary glands lipids of A. americanum may be interpreted as a consequence of intense growth and differentiation of an organ at the centerpiece of a tick's successful parasitization of a vertebrate host. These alterations further show a significant uptake of arachidonic acid at the appropriate time to then be converted to biologically active prostaglandins. Further investigation into the role of prostaglandins in tick feeding and regulation of enzymes involved in prostaglandin synthesis and arachidonic acid release will give added insight into the significance of these findings.

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Table I. Salivary gland mass during feeding of adult *Amblyomma americanum*.

Feeding Stage	Salivary gland Weight (mg)	Feeding Time (days)
Unfed females	0.09 ± 0.01	—
4 - 20 mg	0.27 ± 0.03	4 - 7
20 - 50 mg	0.60 ± 0.13	11 - 14
50 - 100 mg	1.08 ± 0.24	11 - 14
100 - 250 mg	2.21 ± 0.31	11 - 14
>250 mg	1.96 ± 0.40	11 - 14
Replete	1.83 ± 0.32	11 - 15
Virgin females	0.37 ± 0.09	12 - 13
Unfed males	0.05 ± 0.01	—
Fed males	0.08 ± 0.01	11 - 14

Salivary gland mean weights ± SD; n=3. Feeding time ranges given represent length of time on host. Actual time of feeding, especially for males, may be shorter.

Table II. Distribution of arachidonic acid among major phospholipid and neutral lipid classes in salivary glands of Amblyomma americanum females in various stages of feeding

Feeding Stage	PC	PE	PI	FA	TG
Unfed	7.0 ± 1.0	1.0 ± 0.0	1.0 ± 0.0	6.0 ± 0.0	30 ± 5.0
4-20mg	90 ± 40	20 ± 10	ND	ND	ND
20-50mg	160 ± 80	100 ± 70	ND	ND	ND
50-100mg	180 ± 80	110 ± 50	ND	ND	ND
100-250mg	490 ± 340	160 ± 50	ND	ND	ND
>250mg	250 ± 120	100 ± 5.0	ND	ND	ND
Replete	300 ± 100	100 ± 30	ND	ND	ND
Virgins	22 ± 2.0	10 ± 0.0	4.0 ± 0.0	ND	11 ± 0.0

Mean amounts in μg/gland ± SD; n=3 (n=4 for 50-100mg), ND = not detected.

Table III. Increase in salivary gland fatty acids during tick feeding.

Fatty acid	Total amount (μg) Unfed	Maximum	Fold increase
Arachidonic (20:4)	0.04	1.60	40X
Linoleic (18:2)	0.16	3.20	20X
Stearic (18:0)	0.21	2.65	13X
Oleic (18:1)	0.97	10.68	11X
Palmitoleic (16:1)	0.08	0.68	8.5X
Myristic (14:0)	0.13	0.84	6.5X
Palmitic (16:0)	0.42	1.28	3X

Mean amounts in micrograms per salivary gland; n=3.

Figure 1. Total fatty acids in phospholipid class from tick salivary glands at various stages of feeding. Mean μg/gland ± S.D., n=3.

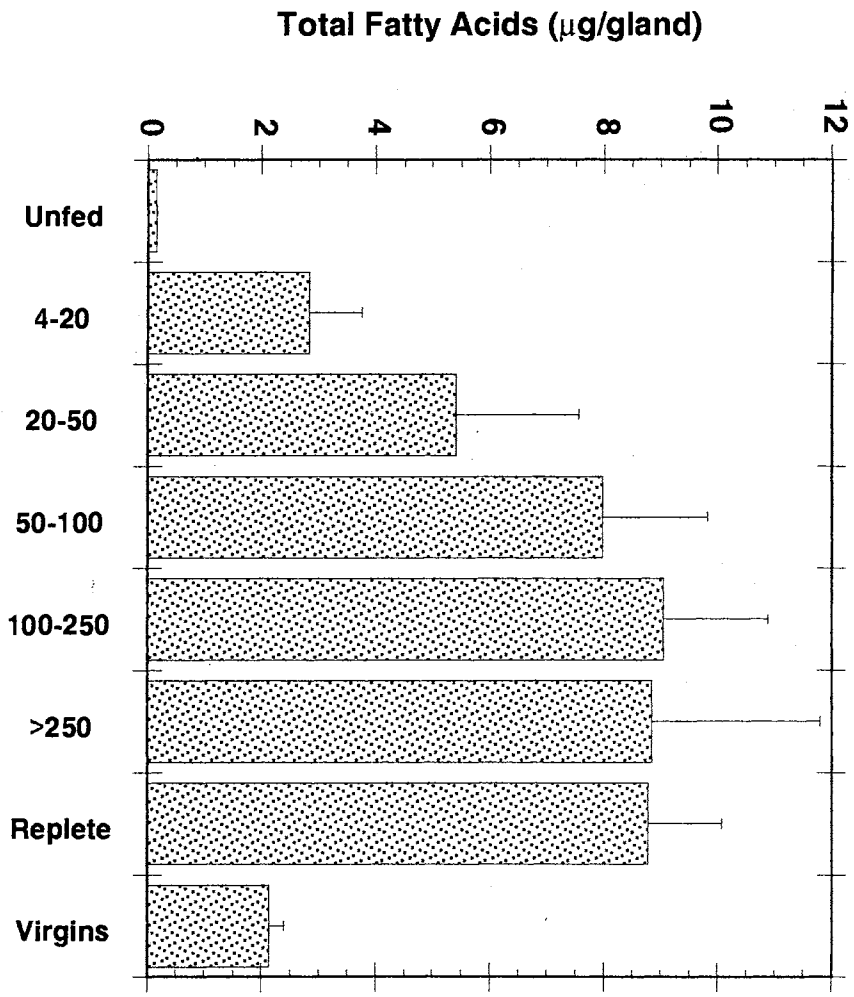

Total Fatty Acids (µg/gland)
0
2
4
6
8
10
12
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 2. Total free fatty acids from tick salivary glands at various stages of feeding. Mean μg/gland ± S.D., n=3.

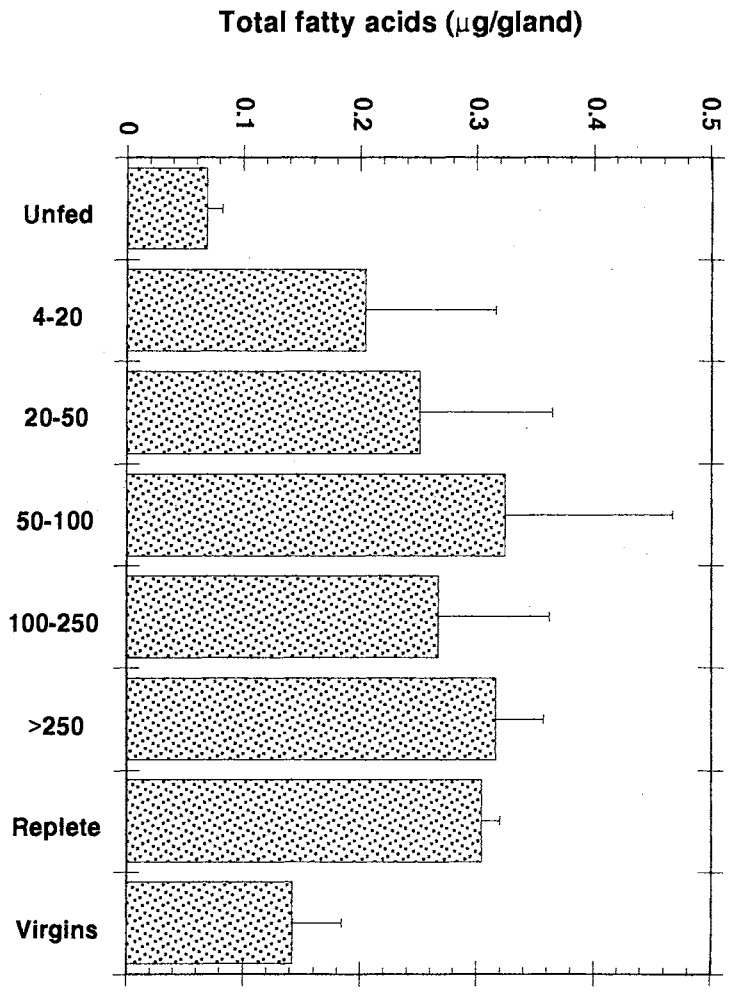

Total fatty acids (μg/gland)
0
0.1
0.2
0.3
0.4
0.5
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 3. Total fatty acids in diglyceride class from tick salivary glands at various stages of feeding. Mean μg/gland $\pm$ S.D., n=3.

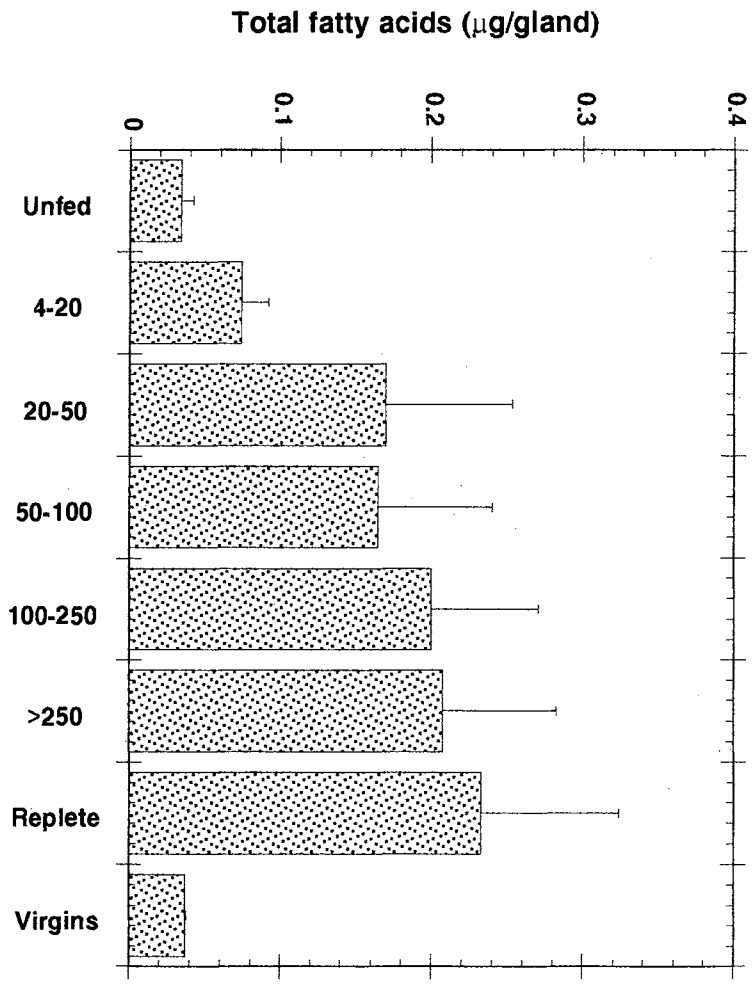

Total fatty acids (μg/gland)
0
0.1
0.2
0.3
0.4
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 4. Total fatty acids in triglyceride class from tick salivary glands at various stages of feeding. Mean μg/gland ± S.D., n=3.

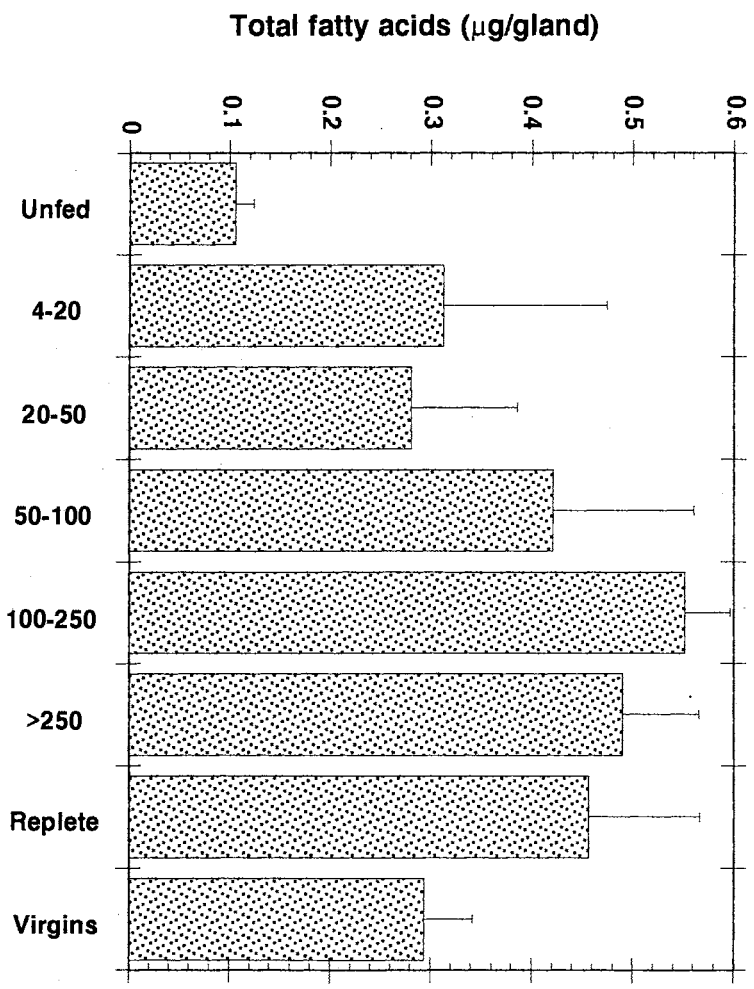

Total fatty acids (µg/gland)
0
0.1
0.2
0.3
0.4
0.5
0.6
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 5. Total fatty acids in sterol ester class from tick salivary glands at various stages of feeding. Mean μg/gland ± S.D., n=3.

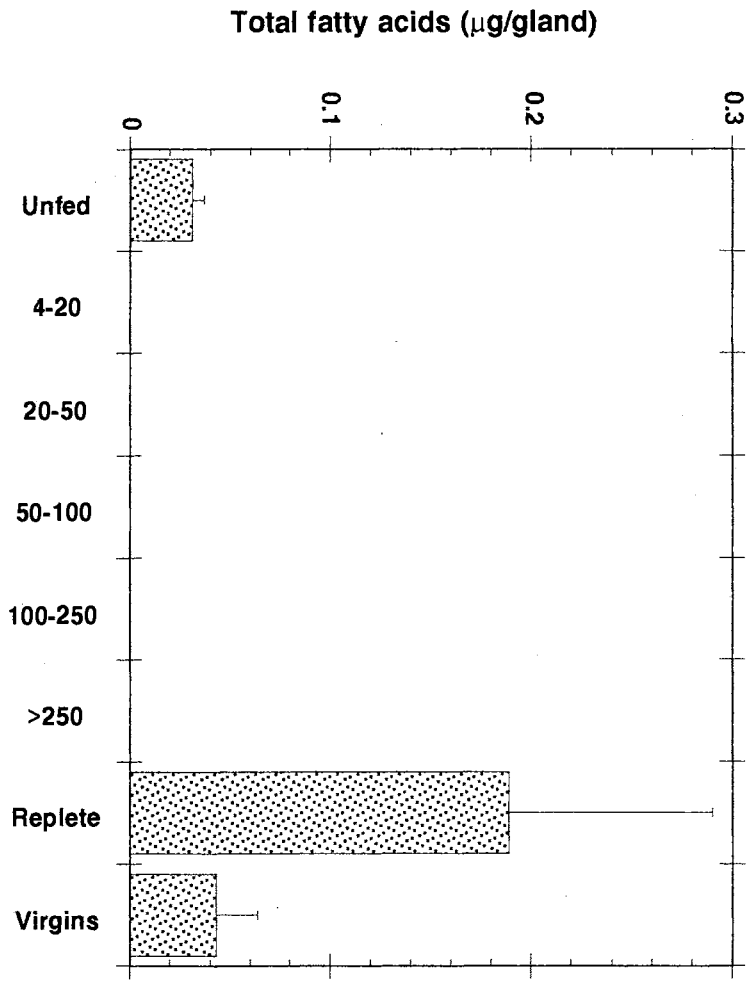

Total fatty acids (μg/gland)
0
0.1
0.2
0.3
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 6. Total fatty acids in phosphatidylcholine from salivary glands at various stages of tick feeding. Mean μg/gland $\pm$ S.D., n=3.

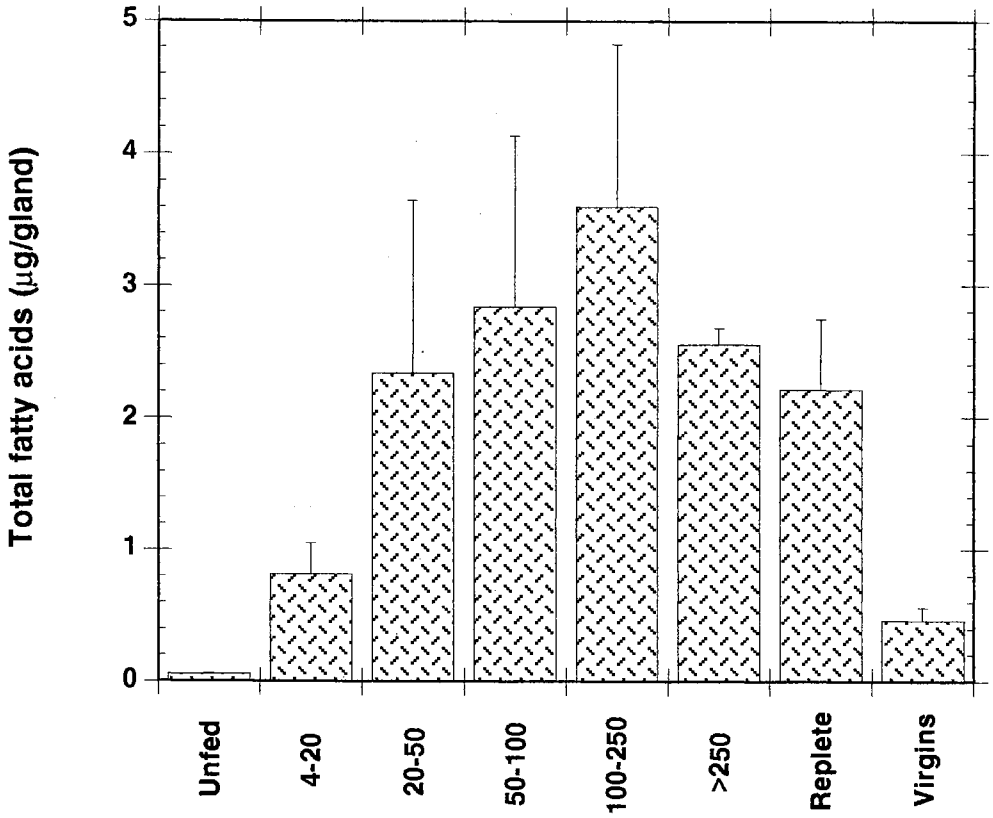

Total fatty acids (µg/gland)
0
1
2
3
4
5
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 7. Total fatty acids in phosphatidylethanolamine from salivary glands at various stages of tick feeding. Mean μg/gland ± S.D., n=3.

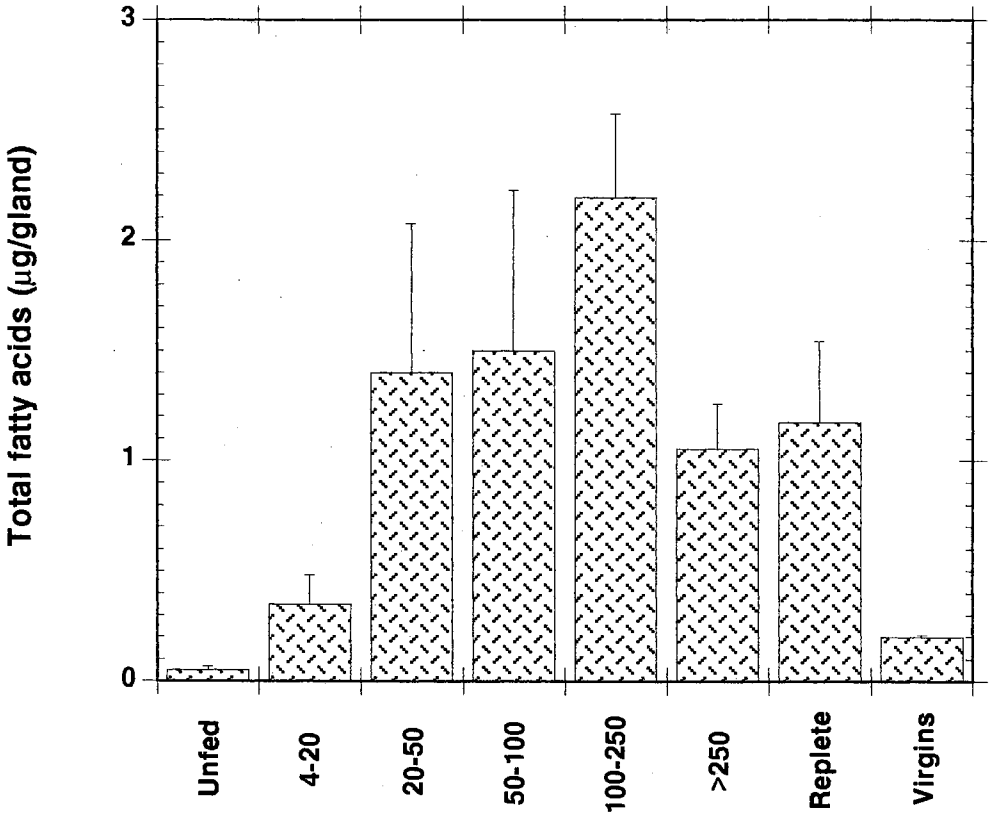

Total fatty acids (µg/gland)
3
2
1
0
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 8. Total fatty acids in phosphatidylinositol from salivary glands at various stages of tick feeding. Mean μg/gland $\pm$ S.D., n=3.

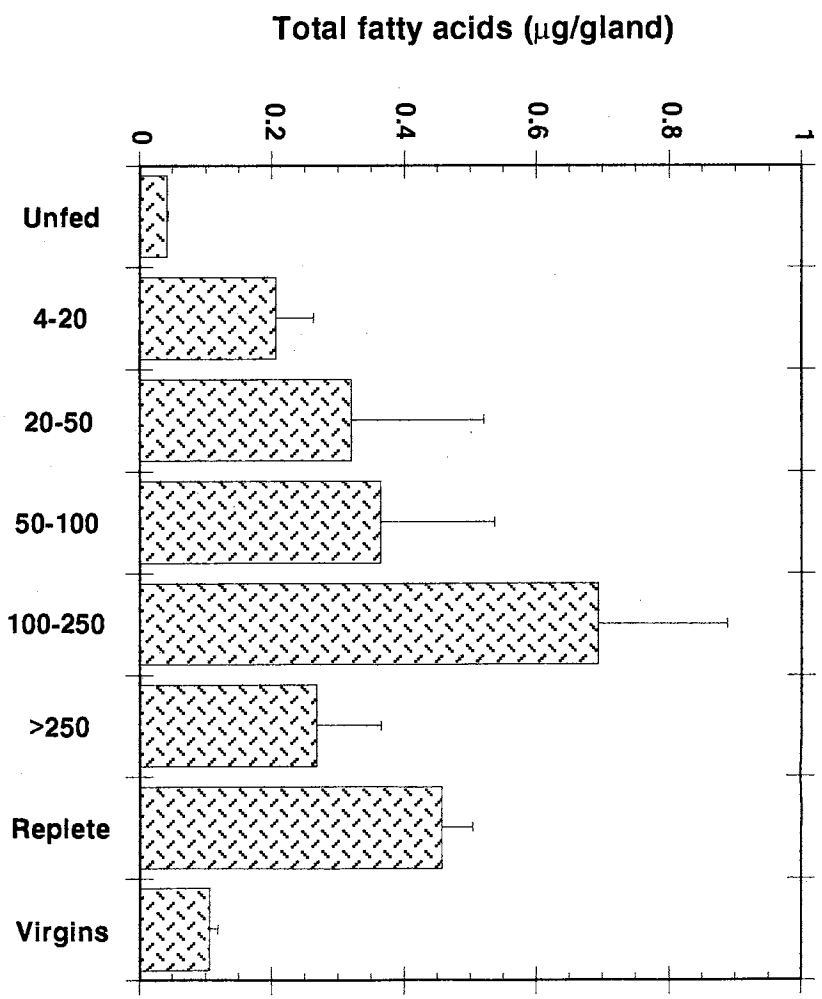

Total fatty acids (μg/gland)
0
0.2
0.4
0.6
0.8
1
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 9. Total fatty acids in lysophosphatidylcholine from salivary glands at various stages of feeding. Mean μg/gland $\pm$ S.D., n=3.

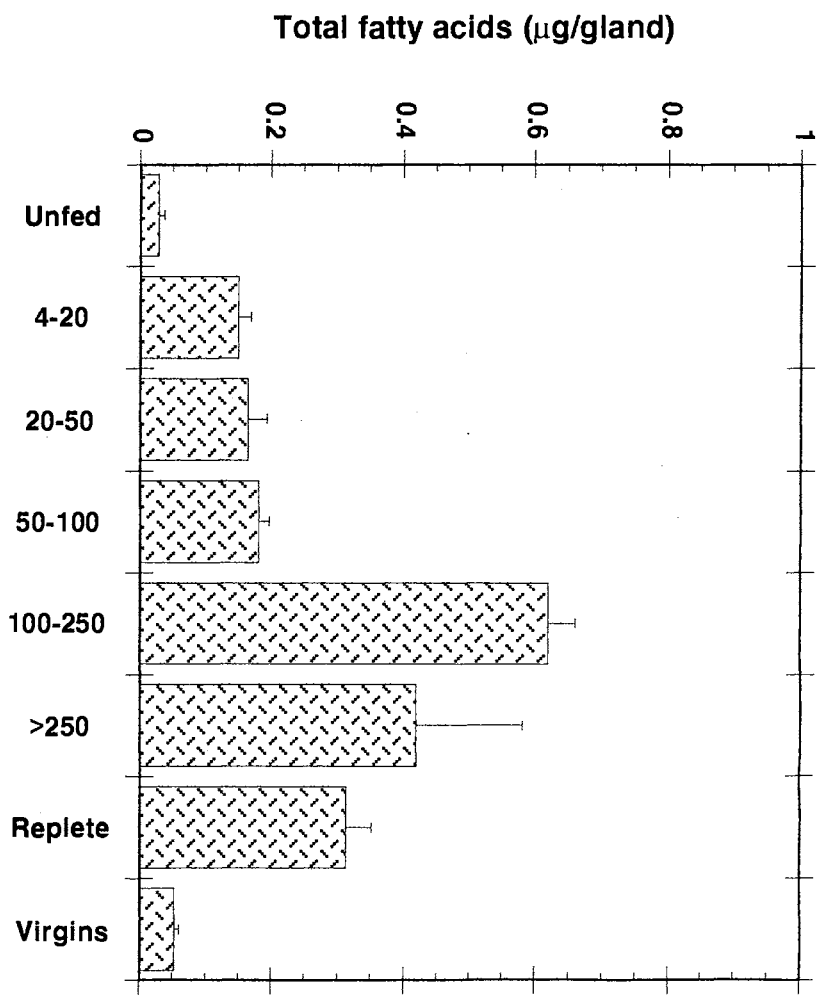

Total fatty acids (μg/gland)
0
0.2
0.4
0.6
0.8
1
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 10. Total fatty acids in cardiolipin from salivary glands at various stages of tick feeding. Mean μg/gland $\pm$ S.D., n=3.

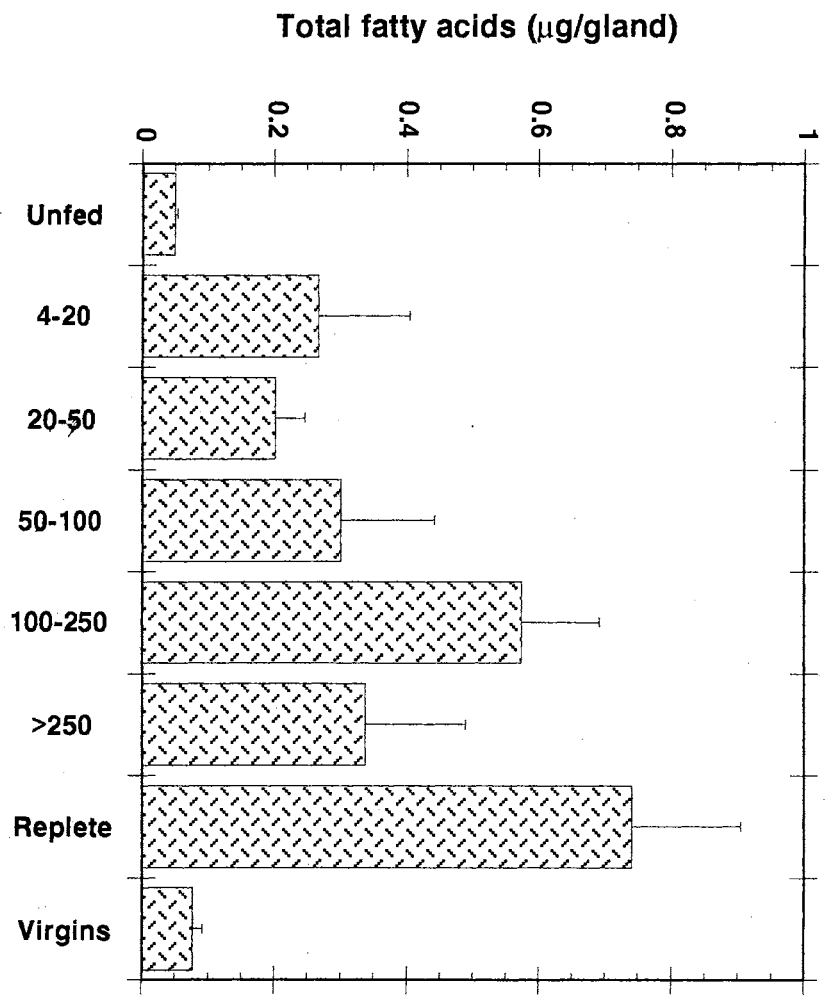

Total fatty acids (µg/gland)
0
0.2
0.4
0.6
0.8
1
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins

Figure 11. Arachidonic acid from total lipid extracts of tick salivary glands from various stages of feeding. Mean percent composition ± S.D., n=3. Numbers above error bars represent microgram amounts of arachidonic acid per salivary gland.

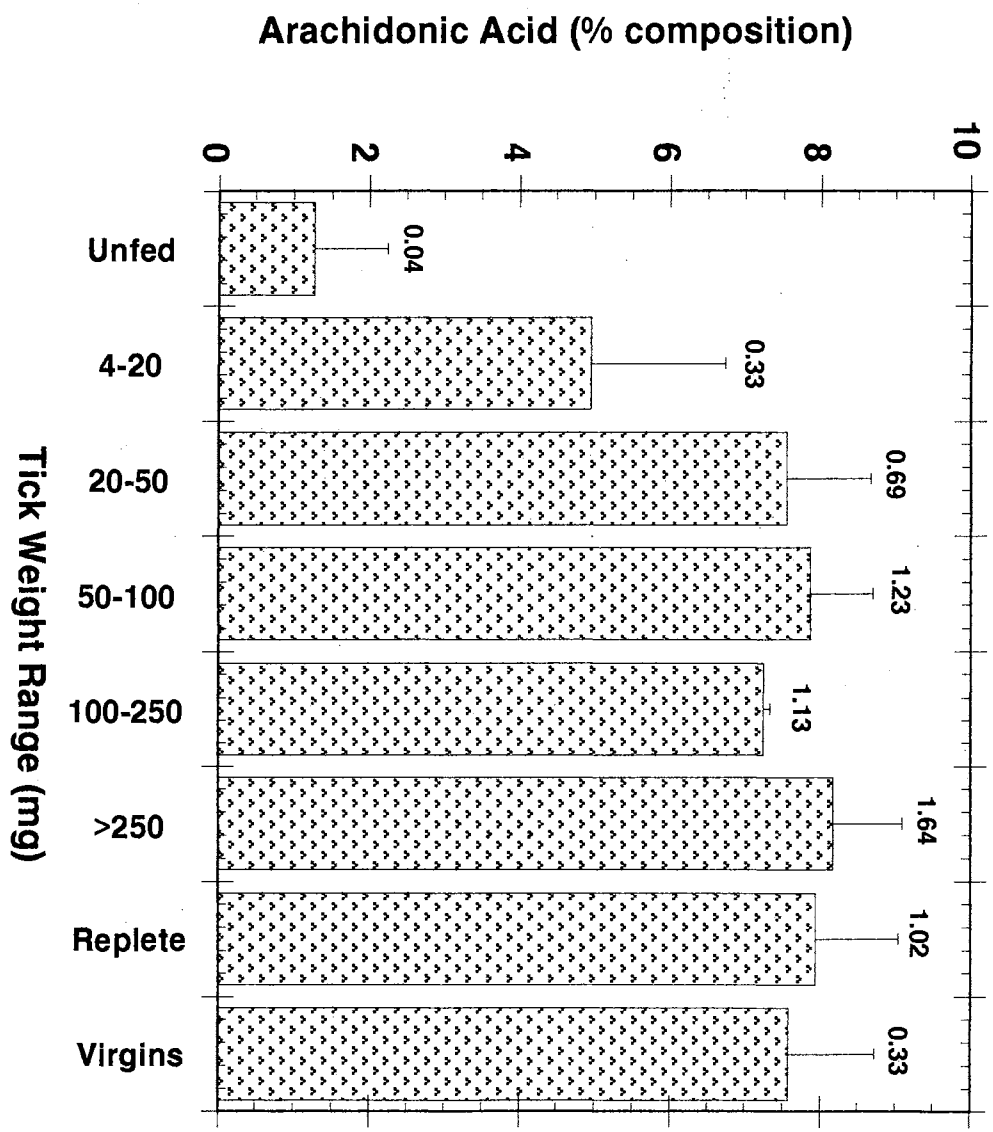

Arachidonic Acid (% composition)
0
2
4
6
8
10
Tick Weight Range (mg)
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins
0.04
0.33
0.69
1.23
1.13
1.64
1.02
0.33

Figure 12. Linoleic acid from total lipid extracts of tick salivary glands from various stages of feeding. Mean percent composition ± S.D., n=3. Numbers above error bars represent microgram amounts of linoleic acid per salivary gland.

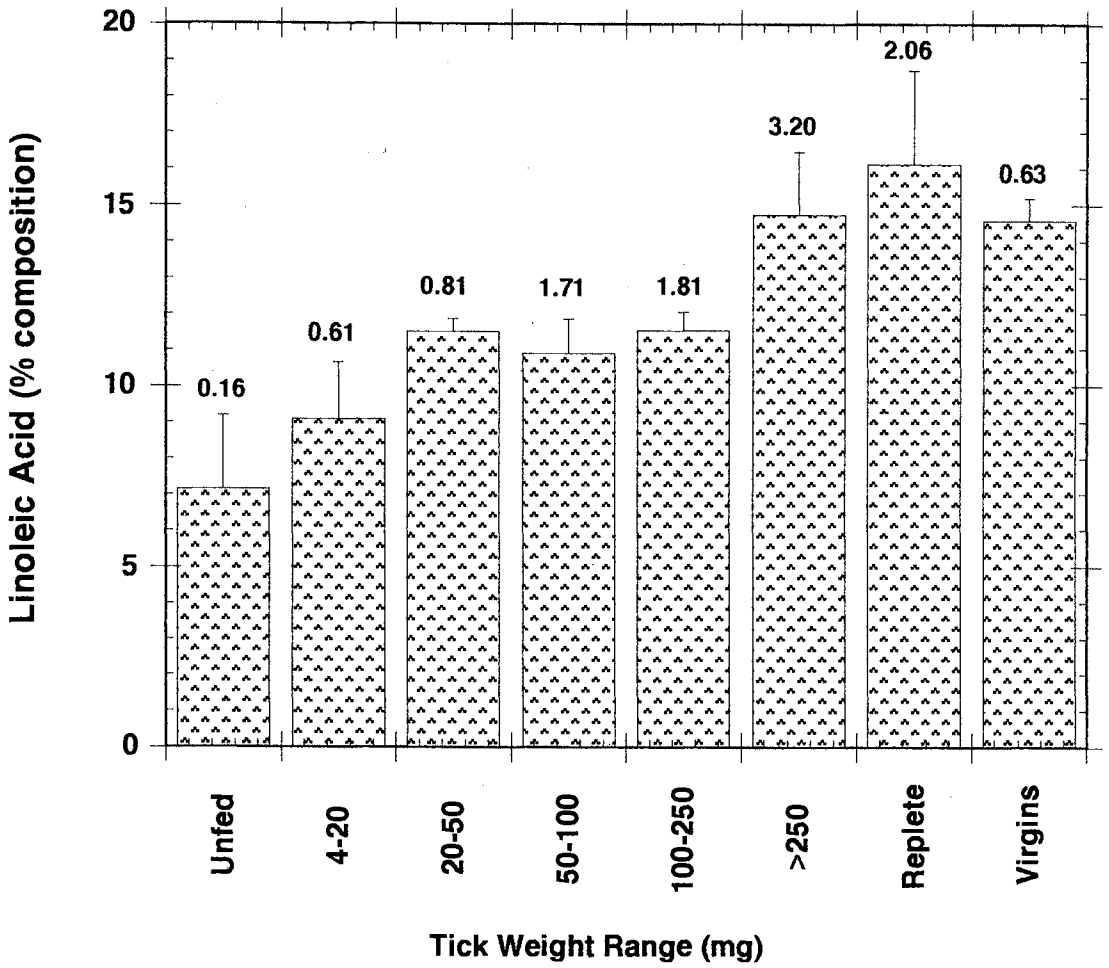

Linoleic Acid (% composition)
20
15
10
5
0
0.16
0.61
0.81
1.71
1.81
3.20
2.06
0.63
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Virgins
Tick Weight Range (mg)

Figure 13. Changes in percent composition of fatty acids from salivary gland total lipid extracts during tick feeding; n=3. A) Saturated fatty acids: Myristic acid; 14:0(■), Palmitic acid; 16:0(▲), Stearic acid; 18:0(◇). B) Unsaturated fatty acids: Palmitoleic acid; 16:1(■), Oleic acid; 18:1(▲), Linoleic acid; 18:2(◇), Arachidonic acid; 20:4(△).

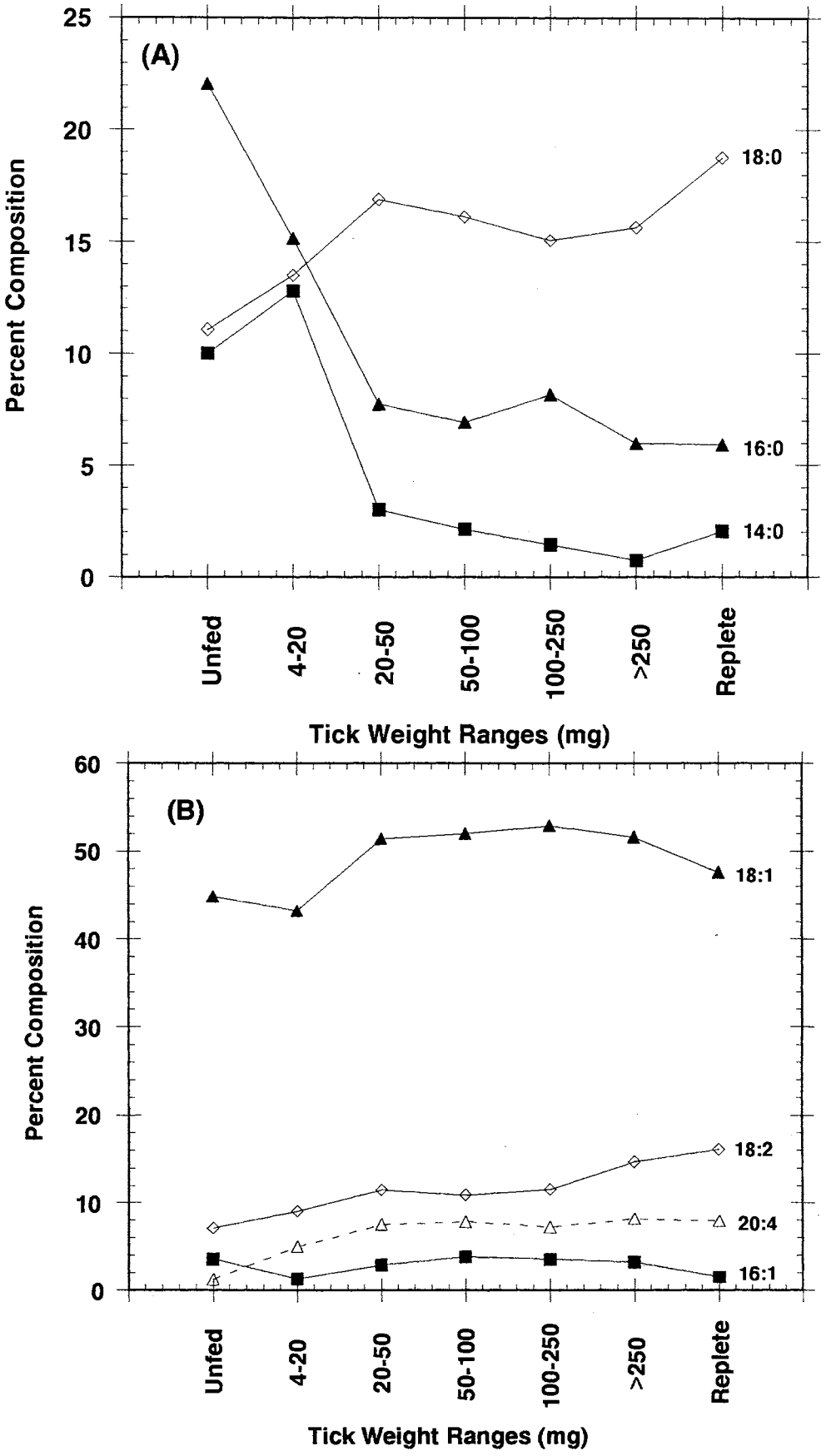
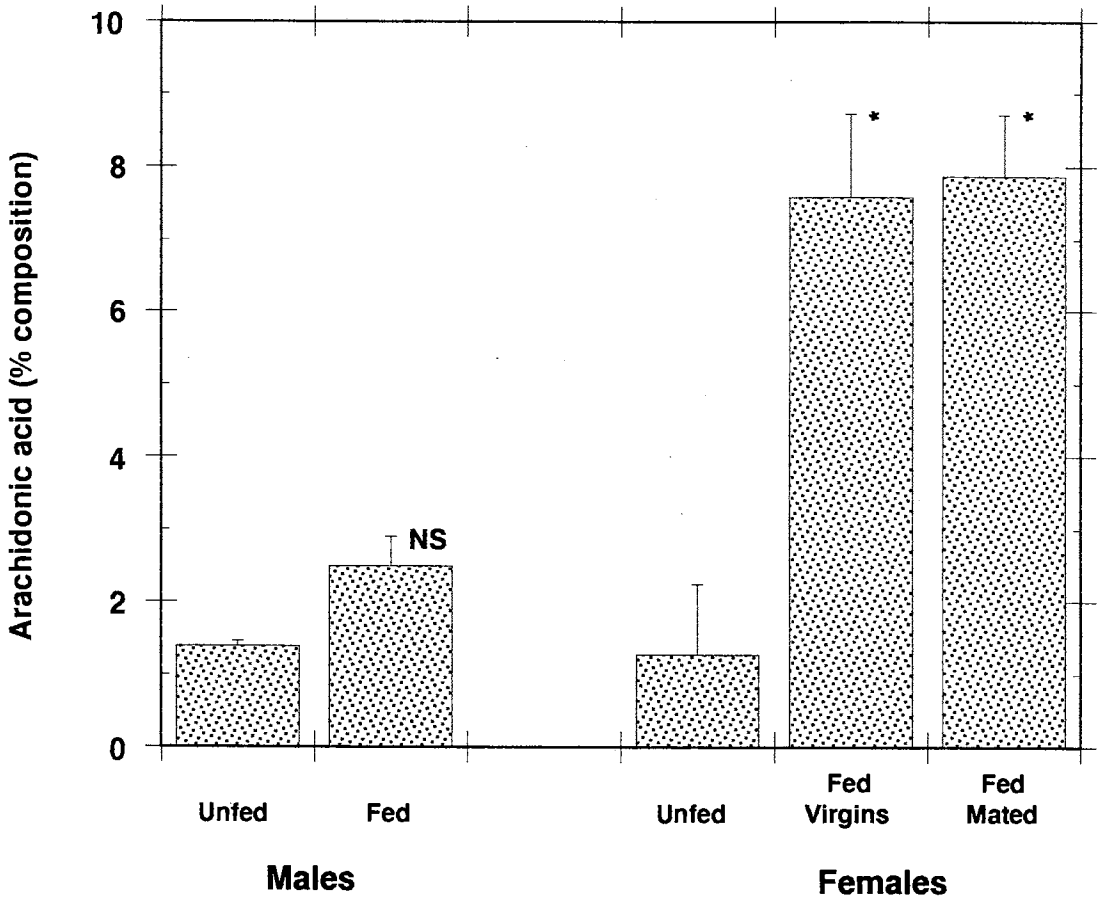

(A)
Percent Composition
25
20
15
10
5
0
18:0
16:0
14:0
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Tick Weight Ranges (mg)
(B)
Percent Composition
60
50
40
30
20
10
0
18:1
18:2
20:4
16:1
Unfed
4-20
20-50
50-100
100-250
>250
Replete
Tick Weight Ranges (mg)

Figure 14. Comparisons of arachidonic acid percent compositions between unfed and fed males, and unfed, fed/virgin, and fed/mated (50-100 mg) lone star ticks; n=3. Means compared by Student's t-test. NS = No significant difference ($p>0.05$) between unfed and fed males. *Significant difference ($p<0.01$) between unfed and fed (virgin and mated) females.


Arachidonic acid (% composition)
10
8
6
4
2
0
NS
*
*
Unfed
Fed
Unfed
Fed Virgins
Fed Mated
Males
Females

PART III

DISTRIBUTION OF ARACHIDONIC ACID IN DIACYL, ALKYLACYL, AND ALKENYLACYL PHOSPHOLIPID SUBCLASSES IN THE SALIVARY GLANDS OF THE LONE STAR TICK

ABSTRACT

The subclass composition of choline- and ethanolamine-containing phospholipids was determined by analysis of acyl-linked fatty acids released by base hydrolysis of diradylglycerobenzoates formed from lone star tick salivary gland diacyl, alkylacyl and alkenylacyl phospholipids. Of the ether-linked phospholipids, the alkylacyl subclass comprises about 9% and the alkenylacyl subclass about 4% of choline phospholipids. Ethanolamine phospholipids consist of about 14% and 13% alkylacyl and alkenylacyl lipids, respectively. Arachidonic acid (20:4) is the most abundant fatty acid released from the acyl linkage of alkylacyl-PC (28% of all fatty acids) and is 17% in alkenylacyl-PC. Alkylacyl-PE is also rich in 20:4 (24%) while the alkenylacyl-PE subclass contains only 9% arachidonic acid. Overall, although the amount of arachidonic acid in ether-linked lipids is relatively high, the majority of the salivary gland arachidonic acid (>83%) is in the diacyl phospholipids because of the higher amounts of this phospholipid subclass in tick salivary glands.

Isolated salivary glands incorporated [^{3}H]-arachidonic acid after 15 min primarily into the diacyl subclass of phosphatidylcholine (PC) > phosphatidylethanolamine (PE), and some into triglycerides. Additional incubation in the

absence of exogenous labeled arachidonic acid for up to 120 min shows a remodeling of ^{3}H-20:4 from PC into PE, and from the diacyl subclass to the alkylacyl subclass in the choline phospholipids.

INTRODUCTION

Lipids that contain ether linkages at positions which more commonly contain the acyl (ester) bond are widely distributed in microorganisms, as well as human and animal tissues (Snyder, 1972). In contrast to the diacyl type of phospholipids, ether-linked phospholipids exist as two distinct types: alkylacyl phospholipids which contain a hydrocarbon chain attached to the glycerol backbone through a saturated ether linkage, and alkenylacyl phospholipids (also called vinyl ethers or plasmalogens) which contain an α,β-unsaturated ether at the sn-1 position (see Figure 1).

The precise function of ether-linked lipids in many biological systems is obscure. However, determination of the structure of platelet-activating factor (PAF; 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) by three independent groups (Benveniste et al., 1979; Blank et al., 1979; Demopoulos et al., 1979) showed an ether-linked phospholipid with biological activity, and studies on these lipids have since progressed at a swift pace.

Most mammalian tissues studied to date have low amounts of alkylacyl and high amounts of alkenylacyl phospholipids, especially in the ethanolamine-containing phospholipids (Horrocks and Sharma, 1972). On the other hand, invertebrate animals other than insects have quite

high amounts of alkylacyl lipids in their total or phospholipid fraction (Sugiura et al., 1992) in addition to high levels of alkenylacyl lipids. Most insect species possess low amounts of both ether-linked phospholipid types (Lambremont and Wood, 1968; Sugiura et al., 1992), although elevated amounts in some species suggest that developmental stage or mode of life may be associated with the lipid profile of an insect (Sugiura et al., 1992).

Recently, analysis of the lipid composition of the salivary glands of the lone star tick, Amblyomma americanum revealed an abundance of arachidonic acid (20:4, n-6) primarily in the sn-2 position of phosphatidlycholine and phosphatidylethanolamine (Shipley et al., 1993). Once released from phospholipids by phospholipase A_2 (PLA_2), arachidonic acid can be converted to biologically active molecules such as prostaglandins, which have been identified in the saliva of at least four species of ticks, including A. americanum (Ribeiro et al., 1992; Sauer et al., 1993). Prostaglandins may play a role in counteracting hemostasis, host immunity and inflammatory responses, which are important for the successful feeding of a hematophagous arthropod (Bowman et al., 1993).

Arachidonic acid made available for prostaglandin biosynthesis may come from any of the three possible phospholipid subclasses. Ether-linked phospholipids have been identified as the primary source of arachidonic acid in neutrophils and mast cells (Chilton and Connell, 1988;

Nakamura et al., 1991). On the other hand, alkylacyl choline phospholipids have been shown to be a less preferred substrate than the diacyl form for phospholipase A_2 from various sources (Waku and Nakazawa, 1972; Colard et al., 1987) including PLA_2 activity in lone star tick salivary glands (Surdick, personal communication).

This present study was performed to identify and quantify the phospholipid subclasses in the salivary glands of female lone star ticks, and to show the distribution of arachidonic acid in each subclass. Isolated salivary glands were also incubated with [^{3}H]-arachidonic acid to assess incorporation of label and subsequent remodelling of arachidonic acid in phospholipid subclasses.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid, L-α-1-palmitoyl-2-[1-^{14}C]-linoleoyl-phosphatidylcholine, 1-arachidonyl-2-[1-^{14}C]-arachidonyl-phosphatidylethanolamine, and 1-hexadecyl-2-[^{3}H]-arachidonyl-phosphatidylcholine were purchased from Dupont-New England Nuclear, Boston, MA. Arachidonyl-PAF standard was acquired from Cayman Chemical, Ann Arbor, MI, and phosphatidylcholine plasmalogen (from beef heart) standard was purchased from Serdary, London, Ontario, Canada. All other lipid standards, phospholipase C (from Bacillus cereus), butylated hydroxytoluene (BHT),

benzoic anhydride, 4-dimethyl-aminopyridine, and other chemicals were acquired from Sigma, St. Louis, MO. Aminopropyl columns were acquired from Analytichem International, Harbor City, CA. Thin-layer chromatography (TLC) plates were from Analtech, Newark, DE (silica gel G and GF preparative plates; 500 μm thickness), and Whatman, Maidstone, England (silica gel 60A preparative plates with preabsorbant zone; 250 μm thickness). Silicic acid (Bio-Sil A; 100-200 mesh) was purchased from Bio-Rad, Richmond, CA. Diethyl ether was obtained from EM Science, Gibbstown, NJ. All other solvents were from Fisher Scientific, Pittsburgh, PA, and were redistilled in glass before use.

Extraction of lipid from tick salivary glands

Lone star ticks, Amblyomma americanum (L.) were reared according to the methods of Patrick and Hair (1975). Adult female ticks were placed on ovine hosts along with male ticks and allowed to feed for several days prior to removal. Unfed ticks were maintained at 27-28°C and 90-100% relative humidity before infestation of the host. Salivary glands were dissected in 0.1 M morpholinopropane sulfonic acid buffer (MOPS, pH 6.8; Needham and Sauer, 1979) with 20 mM EGTA at 4°C, then homogenized in 800 μl buffer. Lipids were extracted from the homogenate by the method of Bligh and Dyer (1959) and stored at -15°C in chloroform with BHT (5 mg/100 ml).

Separation of lipids by TLC

Lipid samples were dried under nitrogen gas, and 50μl of a 1 mg/ml solution of phosphatidylcholine diheptadecanoyl and the same amount of phosphatidylethanolamine diheptadecanoyl was added to each sample as an internal standard. Samples were separated into individual lipid classes by thin-layer chromatography on silica gel GF plates (Analtech) as previously described (Shipley et al., 1993) using the solvent system hexane-diethyl ether-acetic acid (70:30:1 v/v). Plates were visualized under iodine vapor and phospholipids were extracted by the method of Arvidson (1967). Phospholipid samples were chromatographed in chloroform-hexane-methanol-acetic acid-boric acid (40:30:20;10:1.8 v/v/v/v/w; Gilfillan et al., 1983) on Whatman TLC plates with preabsorbant zone, and PC and PE were extracted (Arvidson, 1967).

Preparation of benzoate derivatives and separation of phospholipid subclasses

The PC and PE samples were dried under nitrogen gas, dissolved in 1 ml diethyl ether, and 500 μl 0.1M Tris buffer, pH 7.4, was added. Each sample was then treated with 30 units of phospholipase C (from Bacillus cereus) and incubated in a shaking water bath at 37°C for 2 hours (Mavis et al., 1972). The resulting diradylglycerols were extracted three times with 2 ml diethyl ether. After

evaporation of the ether under nitrogen gas, the diradylglycerols were converted to benzoate derivatives by dissolving in 0.3 ml benzene containing 10 mg benzoic anhydride and 4 mg of 4-dimethyl-aminopyridine (Blank et al., 1984) and standing at room temperature for 1 hour. The samples were then placed in an ice bath and 1 ml 0.1 N NaOH was added slowly to stop the reaction, after which the benzoates were extracted three times with 2 ml hexane.

The benzoate subclasses (diacyl, alkylacyl, and alkenylacyl), were resolved by TLC using the solvent system benzene/hexane/diethyl ether (50:45:4 v/v) on silica gel G plates (Blank et al., 1984) and were visualized with iodine vapor. The products were identified by co-migration with both radiolabeled and cold standards. L-α-1-palmitoyl-2-[1-^{14}C]-linoleoyl-PC and 1-arachidonyl-2-[1-^{14}C]-arachidonyl-PE were labelled diacyl standards, and 1-hexadecyl-2-[^{3}H]-arachidonyl-PC was used as an alkylacyl standard. Additionally, arachidonyl-PAF (alkylacyl), PC plasmalogen (alkenylacyl), and PC and PE (diacyl) cold standards were used. All standards were converted to benzoates by the methods previously described. Plates with radiolabeled standards were run on a radio-TLC scanner (Bioscan, Washington, DC) to discern Rf values for diacyl and alkylacyl subclasses. The silica gel band containing each subclass was scraped into 2 ml ethanol and vortexed, and 2 ml water was added and the mixture extracted (top layer removed) three times with 2 ml hexane.

Preparation of fatty acid methyl esters and analysis by gas chromatography

Each sample was dried under nitrogen and 50 μl of a 1 mg/ml solution of heptadecanoic acid methyl ester (17:0) was added to the alkylacyl and alkenylacyl samples as an internal standard. Each sample was hydrolyzed with KOH and fatty acid methyl esters (FAMEs) were prepared for gas chromatography (GC) analysis as previously described by Shipley et al. (1993). By this procedure all acyl-bound fatty acids were cleaved from the diradylglycerobenzoates. FAMEs were analyzed on an HP 5890GC with flame ionization detector (Hewlett-Packard, Sunnyvale, CA) using a fused silica column (DB-225, 30 m x 0.15 mm; 0.15 μm film thickness; J&W Scientific, Folsom, CA) with chromatographic conditions as described previously (Shipley et al., 1993). Retention times of tick salivary gland FAMEs and a FAME standard mixture were compared.

Incorporation of [^{3}H]-arachidonic acid into tick salivary glands

Isolated salivary glands (80-100) were placed into a microcentrifuge tube and 1 ml of oxygenated 20 mM morpholinopropane sulphonic acid-buffered saline (TS/MOPS; pH 7.0), with 0.1 g/l bovine serum albumin (BSA) was added to the tube, which was centrifuged briefly and the supernatant removed. The glands were transferred in 400 μl of the oxygenated TS/MOPS + BSA to a vial that contained 3

μCi [^{3}H]-arachidonic acid dissolved in 5 μl ethanol and 100 μl oxygenated TS/MOPS + BSA. The glands were incubated with gentle shaking at 37°C for 15 min. The labeling reaction was terminated by the addition of 800 μl ice cold TS/MOPS with 30μl 1 mM cold arachidonic acid added. The glands were centrifuged and the supernatant removed, and then the glands were washed twice with 1 ml TS/MOPS + arachidonic acid and twice with TS/MOPS + BSA to remove any exogenous arachidonic acid.

The labeled salivary glands were dispensed into five equal aliquots in 800 μl oxygenated TS/MOPS buffer. One aliquot was immediately homogenized and lipids were extracted (Bligh and Dyer, 1959), and the remaining samples were further incubated in buffer for 15, 30, 60, and 120 min respectively before homogenization and lipid extraction.

Separation of radiolabeled lipid classes and subclasses

To identify lipid fractions containing radioactivity, lipids were separated by TLC using hexane/diethyl ether/acetic acid (70:30:1) as previously described and plates were run on a radio-TLC scanner. In subsequent experiments, the extracted lipids were fractionated into phospholipids, free fatty acids, and neutral lipids by solid phase extraction using aminopropyl columns (Kaluzy et al., 1985). Briefly, samples were dried under nitrogen and resuspended in 50 μl hexane. Aminopropyl columns were

washed with 2 ml hexane, and then lipid samples were loaded onto columns, which were washed with 4 ml chloroform:2-propanol (2:1) to elute neutral lipids, 4 ml 2% acetic acid in diethyl ether to elute free fatty acids, and then 6 ml methanol to elute phospholipids.

The amount of label in a small aliquot (1%) of the phospholipid fraction, along with all of the neutral lipid and free fatty acid fractions, was determined by liquid scintillation counting after evaporation of solvents. The remainder of the phospholipid fraction was separated into composite phospholipids by TLC as previously described. Plates were scanned to identify phospholipid fractions containing radioactivity. These fractions were extracted (Arvidson, 1967) and 1% of each sample was placed in a vial for scintillation counting.

The phospholipids (PC and PE) were converted to benzoates as described earlier and subclasses were separated on silica gel G TLC plates (Blank et al., 1984) using benzene/hexane/diethyl ether (50:45:4 v/v). Plates were scanned to visualize incorporation of ^{3}H-20:4 and then appropriate bands were scraped and the amount of label in each subclass determined by liquid scintillation counting.

RESULTS

Phospholipid subclasses composition in tick salivary glands

The subclass composition of choline- and ethanolamine-

containing phospholipids was quantitated by determining the amounts of acyl-linked fatty acids released by base hydrolysis from the sn-2 position of alkylacyl and alkenylacyl phospholipids, and from the sn-1 and sn-2 position of the diacyl molecules, based on the amount of 17:0 internal standard. Table 1 shows the percent composition of the three subclasses in both slow (<200 mg) and fast feeding female ticks. The diacyl subclass is the most common form in PC and PE from both feeding stages. Ether-linked lipids comprise 13% of the choline phospholipids and about 28% of the ethanolamine phospholipids in slow feeders. Fast feeding ticks also show a higher level of alkylacyl and alkenylacyl types in the ethanolamine phospholipids. The alkenylacyl subclass is the least abundant form in all phospholipids from both stages of feeding, yet ethanolamine phospholipids have three times more alkenylacyl lipids than choline phospholipids.

Little difference is noted when the two feeding stages of the tick are compared. The most notable difference is in the ether-linked subclasses of ethanolamine phospholipids, which show a slight decrease in percent composition as the tick feeds.

Fatty acid composition of phospholipid subclasses

Fatty acids released by base hydrolysis from choline diradylglycerobenzoates and assessed by gas chromatography

are shown for slow feeding ticks in Figure 2. These fatty acids represent those attached to glycerol through the acyl bond (i.e. no ether bonds at the sn-1 position of alkylacyl and alkenylacyl subclasses were disrupted by the preparation of FAMEs). In the diacyl PC fraction, stearic (18:0) and oleic (18:1) acids were the most abundant, followed by palmitic (16:0) and arachidonic (20:4) acids (Fig. 2A). The remaining fatty acids, myristic (14:0), palmitoleic (16:1), linoleic (18:2), and arachidic (20:0) acids together comprised less than 7% of the total. Arachidonic acid is the most abundant fatty acid (28%) in alkylacyl-PC (Fig. 2B). The levels of 18:1, 18:0, and 16:0 are also high, while no 16:1 or 20:0 was detected in this subclass. In alkenylacyl-PC fraction (Fig. 2C), the proportion of 20:4 is lower (17%) and 18:1 is the primary fatty acid present. In statistical analysis (Tukey's procedure) there were no significant differences in percentages of arachidonic acid among the three PC subclasses at P=0.05.

Figure 3 shows the fatty acid profile of subclasses from ethanolamine phospholipids from salivary glands of partially-fed ticks. The diacyl-PE fraction shows a large percentage of 18:0 (Fig. 3A), which at 48.5% is equal to the amounts of 18:1 and 20:4 combined. In the alkylacyl subclass (Fig. 3B), there is virtually no difference in the amounts of 16:0, 18:0, 18:1 and 20:4. Arachidonic acid comprises quite a large proportion (23.7%) of the

alkylacyl-PE fatty acids, and is significantly higher than the level of 20:4 in alkenylacyl-PE at 9.4% (Fig. 3C). No 16:1 or 20:0 was detected in the ethanolamine-containing phospholipid subclasses in this study.

The composition of fatty acids in phospholipid subclasses from salivary glands of fast feeding ticks was basically similar to that of slow feeding ticks (data not shown). The major fatty acids in all subclasses were 16:0, 18:0, 18:1, and 20:4. One difference in the fatty acid profile of engorged ticks is that the percent composition of 20:4 was generally lower in each individual subclass. Arachidonic acid made up 17.6%, 23.7%, and 9.4% of the diacyl, alkylacyl, and alkenylacyl subclasses of choline phospholipids respectively, and 15.1%, 16.6%, and 13.1% of the ethanolamine-containing subclasses respectively in fast feeding ticks.

Distribution of arachidonic acid in phospholipid subclasses

From the fatty acid GC analysis the distribution of arachidonic acid within the three subclasses and the amounts in micrograms per salivary gland are shown in Table 2. Most of the arachidonic acid (>83%) is found in the diacyl subclass in all fractions. In slow feeding ticks 12.5% of the 20:4 in the choline-containing phospholipids was found in ether-linked lipids, primarily the alkylacyl form, which had 3-fold more arachidonic acid than the alkenylacyl subclass. The arachidonic acid in

ethanolamine-containing subclasses was similarly distributed in slow feeders, with 16.3% of the 20:4 in ether-linked fractions.

The choline-containing phospholipids of fast feeding tick salivary glands show 13.3% of the 20:4 to be in the ether-linked phospholipids, yet the great majority of that is in the alkylacyl subclass, which has over 7-fold more 20:4 than the alkenylacyl fraction. The ethanolamine-containing phospholipids of fast feeders had 16.8% of its arachidonic acid in the ether-linked lipids, with a higher percentage (7.1%) in the alkenylacyl subclass than any other sample.

Incorporation of [^{3}H]-arachidonic acid into tick salivary gland lipids

After 15 min of incubation with labeled arachidonic acid, 65% of the added label was incorporated into the phospholipid class, triglycerides accounted for 10% and 25% was present as free arachidonic acid (data not shown). After an additional 15 min incubation in the absence of added radioactivity, a decrease in the percentage of free arachidonic acid was seen as label was redistributed to the phospholipid fraction, which rose to 75% of the radioactivity (data not shown). There was no further change in percentage of label among phospholipids, triglycerides, and free arachidonic acid during the remainder of the 120 min incubation.

The only phospholipids incorporating [^{3}H]-20:4 were PC, which amassed 76% of the radioactivity after a 15 min pulse, and PE with 24% (depicted as 0 min in Fig. 4). Upon further incubation of tick salivary glands after removal of exogenous arachidonic acid, remodeling of 20:4 is seen as PE increases to 29.5% of the radioactivity after 15 min. After 120 min of incubation PE increased to 43%, with PC decreasing to 57% of labeled arachidonic acid in phospholipids.

Distribution of labeled arachidonic acid among PC and PE subclasses

After the initial 15 min incubation of tick salivary glands with [^{3}H]-arachidonic acid, most of the label incorporated into phospholipids was present in the diacyl subclass (93-96%). Figure 5 shows the incorporation of arachidonic acid in diacyl-PC as a function of incubation time. The percentage of radioactivity after a 15 min pulse (96%) declined in a significantly linear manner ($r= -0.787$, $P<0.001$) with incubation time to about 93% after 120 min. A concomitant significant linear increase ($r= 0.793$, $P<0.001$) in radioactivity in alkylacyl-PC from 4% to over 6% was noted (Fig. 6). Alkenylacyl-PC showed less than 1% of the radioactivity and did not change during the 120 min incubation (Fig. 6).

More variation was seen in the three experiments for the PE subclasses, and no apparent remodeling among the

three subclasses was seen. Diacyl-PE accounted for over 94% of the label after the 15 min pulse and no significant decrease was seen after chasing for 120 min (Fig. 7). Alkylacyl-PE contained about 5% and alkenylacyl-PE just under 1% of the label, and no redistribution was apparent after 120 min (Fig. 8).

DISCUSSION

Previous studies have shown that ether-linked phospholipids, particularly alkenylacyl ethanolamine phospholipids, are found in a variety of lower animals. Thompson (1972a,b) reviewed the occurrence of ether-linked lipids in protozoa and molluscs, and the distribution in various invertebrates was reviewed by Horrocks and Sharma (1982) and Chapelle (1987). Lambremont and Wood (1968) investigated the occurrence of alkylacyl and alkenylacyl in the phospholipids of the American cockroach, Periplaneta americana, the boll weevil, Anthonumus grandis, and the tobacco budworm, Heliothis virescens. Recently Sugiura et al. (1992) investigated the levels of the three phospholipid subclasses in 28 species of various invertebrates, including 9 insect species.

The present study has revealed the presence of alkylacyl and alkenylacyl phospholipids in choline and ethanolamine phospholipids from the salivary glands of Amblyomma americanum, the first such investigation of a blood-sucking arthropod. The diacyl subclass is the

predominant form existing in tick salivary gland phospholipids. The choline phospholipids contained twice the alkylacyl as compared to alkenylacyl phospholipids, while the levels of two ether-linked phospholipids were nearly equal in the ethanolamine fractions from the salivary glands.

As compared to studies of whole body tissues from most invertebrate animals of lower phyla, there are low amounts of ether-linked phospholipids in tick salivary glands. Various molluscs and echinoderms have very large proportions of plasmalogens, up to 99.2% of the ethanolamine phospholipid class (Horrocks and Sharma, 1982). Most invertebrates, especially of lower phyla, contained large amounts of alkylacyl-PC and alkenylacyl-PE in their tissues (Sugiura et al. 1992). Up to 81.8% of the choline phospholipids found in the sponge, Halichondria japonica, was the alkylacyl type, and as much as 88.7% of the ethanolamine phospholipids were the alkenylacyl type in the clamworm, Marphysa sanguinea. It may be a unique feature of marine animals from lower phyla to have large amounts of alkylacyl-PC in their tissues (Sugiura et al., 1992). The sole member of Arachnida in this study, the spider Grammostola sp., had an ether-linked PC composition of 19.3%, nearly all of this being alkylacyl-PC, and an ether-linked PE composition of 48.6%, with a near equal distribution between alkyl and alkylenyl types.

Insects, however, were characterized as containing

small amounts of ether-linked phospholipids in whole body tissues compared to other invertebrates, and the levels of these lipids in tick salivary glands are higher than amounts found in most insects. Ether-linked phospholipids comprised 0.9%, 6.5%, and 4.4% of total phospholipids from the adult tobacco budworm, boll weevil, and female American cockroach respectively (Lambremont and Wood, 1968). The alkenylacyl subclass was the primary ether-linked phospholipid in all developmental stages of the tobacco budworm, while the dominant form in the cockroach and boll weevil were alkylacyl phospholipids. In further studies, most of the ether-linkages in the tobacco budworm were in ethanolamine phospholipids (Lambremont, 1972a,b). Most of the insects investigated by Sugiura et al. (1992) contained less than 3% ether-linked PC. Three insects in this study were exceptional in having high levels of alkylacyl phospholipids: the predacious diving beetle, Cybister japonicus, the water scorpion, Laccotrephes japonensis, and the larva of the beetle, Allomyrina dichotoma. The habitat of these insects (living in soil or water) more closely resembles invertebrates of lower phyla and may be a reason for similar lipid profiles (Sugiura et al., 1992). The reason for elevated amounts of alkylacyl phospholipids in the beetle larva is unclear, since larvae of other insects studied contained only negligible amounts of alkylacyl phospholipids (Lambremont, 1972b; Sugiura et al., 1992).

The major fatty acids found in all subclasses of

phospholipids in tick salivary glands were 16:0, 18:0, 18:1, and 20:4, which differs somewhat in comparison to the composition of most mammalian ether-linked lipids. The sn-2 position (acyl bond) of fatty acids with 1-ether-linkages contained various amounts of 16:0, 18:0, 18:1, and 20:4, and sometimes high levels of 18:2, 22:4, 22:5, and 22:6 in mammalian tissues (Horrocks, 1972). Most of the ether-linked phospholipids from mammalian tissues have a major portion of unsaturated fatty acids (>90%) at the acyl bond, while a surprisingly high level of saturated fatty acids (about 50%) occupy that position in tick salivary glands. Furthermore, oleic acid (18:1) and linoleic acid (18:2) are seen in smaller percentages in this study compared to earlier studies, which shows 18:1 as the major fatty acid in tick salivary gland phospholipids (Shipley et al., 1993), and reasons for this difference are unknown. In Shipley et al. (1993) only 15% of 18:0 is found in the sn-2 position of PC, yet 18:0 comprises 20-29% of the sn-2 position of ether-linked phospholipids. In studies of the composition of fatty acids at the sn-1 position of mammalian ether-linked phospholipids (alkyl and alk-1-enyl bonds), the major fatty acids were 16:0, 18:0, and 18:1 (Horrocks, 1972).

Most of the fatty acid compositions from ether-linked lipids in invertebrates have been reported in lower animals, and the profiles vary greatly with species and tissues studied. The ethanolamine plasmalogens from the

earthworm nervous tissue were primarily 18:0, (Okamura et al., 1985) a major fatty acid in the tick salivary glands, while some marine invertebrates tend to have high amounts of long chain (C-20-26) polyunsaturated fatty acids in their alkenylacyl-PE fraction (Horrocks and Sharma, 1982; Koizumi et al., 1990).

Experiments in this study where salivary glands were pulse labeled with [^{3}H]-arachidonic acid followed by incubation in buffer without labeled 20:4 suggests that there is a rapid remodeling of arachidonate from PC to PE. The transfer of label slows after 60 min, and the two have nearly equal amounts after 120 min. This is consistent with experiments in human neutrophils (Chilton and Murphy, 1986) and mouse bone marrow-derived mast cells (Fonteh and Chilton, 1992), which imply that arachidonate initially incorporated into PC is remodeled into various PE subclasses. It cannot be determined from this present study, however, if the changes in percent of radioactivity between the two phospholipid molecules represents a true transfer of arachidonate from PC to PE. It is possible that free arachidonate, which decreases during the incubation, may be a source of label for PE. However, the fact that there is no increase in free 20:4 during the 120 min chase is indicative of a direct transfer between the two phospholipids (MacDonald and Sprecher, 1991).

This study has demonstrated an apparent transfer of labeled arachidonic acid from the diacyl to the alkylacyl

subclass of PC. Time-dependent changes in the percent of radiolabeled arachidonate among phospholipid subclasses have been studied in inflammatory cells such as human neutrophils (Tou, 1984; Chilton and Murphy, 1986), rabbit alveolar macrophages (Sugiura et al., 1984), and mouse bone marrow-derived mast cells (Fonteh and Chilton, 1992). In each of these studies a similar loss of radioactivity in diacyl-PC coincides with a gain in alkylacyl-PC. The alkenylacyl-PC subclass in tick salivary glands went unchanged during further incubation, which is consistent with most of the studies with inflammatory cells. No significant remodeling of arachidonate was seen in subclasses of PE in this present study, yet it is ether-linked ethanolamine phospholipids that receive much of the increase in label in studies with inflammatory cells. Over 70% of the label in PE was in the alkenylacyl subclass after a 120 min chase in macrophages (Sugiura et al., 1984), and a large amount was incorporated into alkenylacyl-PE in neutrophils as well (Tou, 1984; Chilton and Murphy, 1986).

Regarding the physiological roles of ether-linked lipids, studies show that alkylacyl phospholipids are stable to phospholipase A_1, and are a less preferred substrate than the diacyl form for phospholipase A_2 and phospholipase C from several bacteria and venoms (Waku and Nakazawa, 1972). However, some isoforms of PLA_2 are more specific for ether-linked phospholipids (Loeb and Gross,

1986), and in this present experiment PLC from B. cereus was effective on all subclasses of phospholipids. Still, it is possible that ether-linked phospholipids when bound in the plasma membrane may protect cells from lipolytic enzymes. Studies show that ether lipids may play a role in ionic regulation and membrane permeability as well. Paltauf (1983) compared the permeabilities of unilamellar and multilamellar membranes that consisted of ether or ester lipids and found higher packing densities and a lower dipolar surface potential in membranes high in ether lipids. Permeability of chloride anions is higher when vesicles have ether-linked phospholipids, yet hydrophobic anions penetrate diacyl phospholipid membranes faster. Alkylacyl-PC also may be important as a stored precursor of PAF, which has emerged as the most potent lipid mediator known with a host of functions such as platelet aggregation, activation of protein kinase C, and inhibition of interleukin-2 secretion (Snyder, 1990). The presence of PAF has been demonstrated in lower animals, and PAF-like lipid was found in all species of insects investigated by Sugiura et al. (1992). The physiological roles of PAF in lower animals in unclear.

The functions of ether-linked phospholipids in the salivary glands of the lone star tick are unknown at this time. Recently a micromolar calcium-sensitive PLA_2 activity was found in the salivary glands (Sauer, et al., 1993) and alkylacyl-PC was shown to be a less preferred substrate

than diacyl-PC (Surdick, personal communication). Fatty acids associated with ether-linked phospholipids may thus be less likely hydrolyzed by PLA_2. The initial step in the production of prostaglandins and other eicosanoids is the liberation of arachidonic acid from the sn-2 position of phospholipids by PLA_2 (Holtzman, 1991). But with the relatively small percentage of arachidonic acid present in ether-linked phospholipids in tick salivary glands, this does not represent a large protected reserve. Determination of the precise physiological functions of ether-linked lipids will bring insight into the consequences of these findings.

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Table 1. Relative proportions of diacyl, alkylacyl, and alkenylacyl subclasses of choline- and ethanolamine-containing phospholipids in salivary glands from slow and fast feeding lone star ticks

Phospholipid subclass	Slow feeders	Fast feeders
Choline phospholipids		
Diacyl	87.1 ± 4.1	87.3 ± 3.6
Alkylacyl	8.5 ± 2.7	8.9 ± 2.9
Alkenylacyl	4.4 ± 1.4	3.8 ± 0.7
Ethanolamine phospholipids		
Diacyl	72.1 ± 7.7	76.7 ± 5.8
Alkylacyl	14.5 ± 2.4	12.8 ± 0.7
Alkenylacyl	13.3 ± 5.3	10.5 ± 5.6

Percent composition based on analysis of fatty acids ± SD; n = 3.

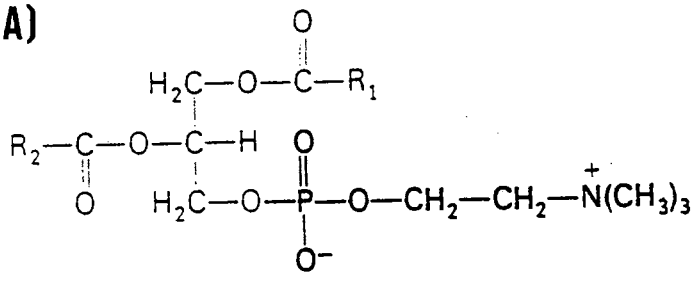
Table 2. Distribution of endogenous arachidonic acid among phospholipid subclasses in salivary glands from slow and fast feeding lone star ticks

Phospholipid subclass	Slow feeders		Fast feeders	
	Percent distribution	Micrograms per gland	Percent distribution	Micrograms per gland
Choline phospholipids				
Diacyl	87.5 ± 3.9	0.33 ± 0.24	86.7 ± 5.1	0.26 ± 0.14
Alkylacyl	9.7 ± 3.1	0.03 ± 0.01	11.6 ± 4.6	0.03 ± 0.01
Alkenylacyl	2.8 ± 0.8	0.009 ± 0.004	1.7 ± 0.7	0.004 ± 0.001
Ethanolamine phospholipids				
Diacyl	83.7 ± 5.1	0.16 ± 0.12	83.2 ± 6.6	0.23 ± 0.14
Alkylacyl	12.5 ± 4.1	0.02 ± 0.01	9.7 ± 5.3	0.02 ± 0.01
Alkenylacyl	3.8 ± 1.1	0.007 ± 0.002	7.1 + 6.6	0.01 ± 0.01

Mean ± S.D.; n = 3.

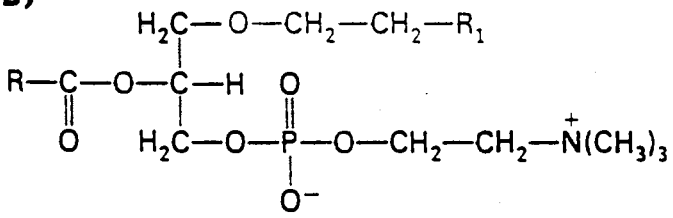
Figure 1. Molecular structures of A) diacyl, B) alkylacyl, and C) alkenylacyl subclasses of choline-containing phospholipids.

A)



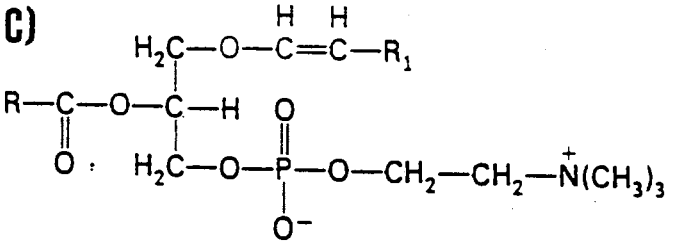
Phosphatidyl choline

B)



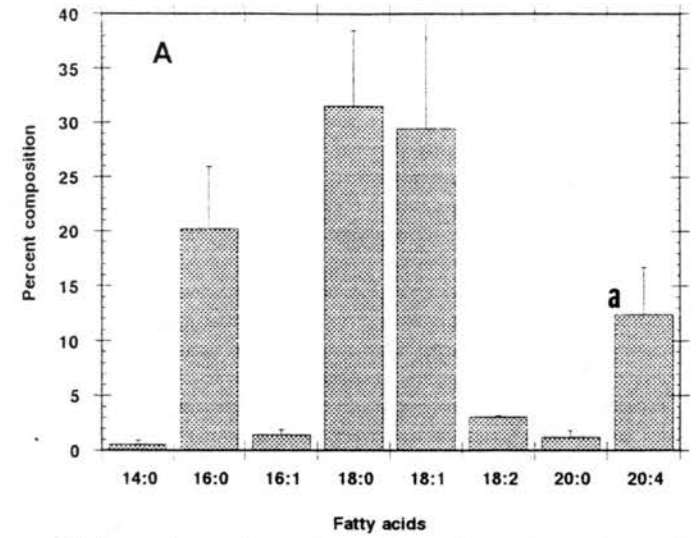
1-Alkyl-2-acyl-phosphatidyl choline
(An ether phospholipid)

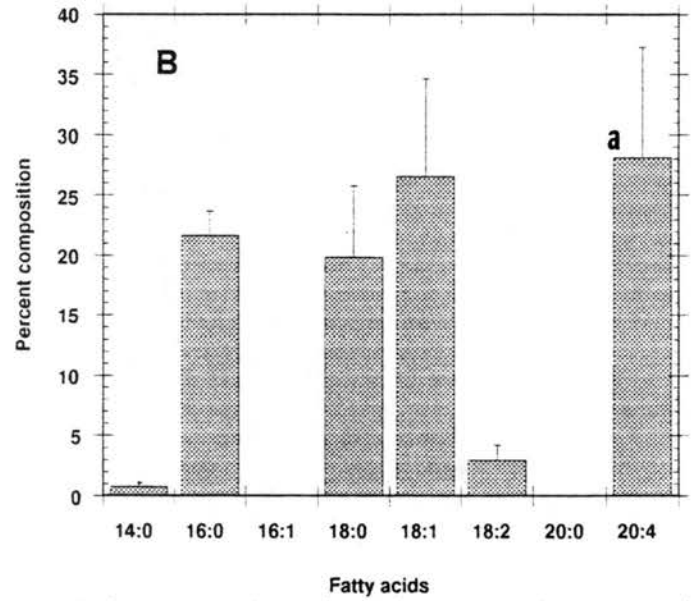
C)



Phosphatidal choline
(A plasmalogen)

Figure 2. Fatty acid composition of (A) diacyl, (B) alkylacyl, and (C) alkenylacyl subclasses of choline phospholipids in salivary glands of slow feeding lone star ticks. Mean percent composition ± S.D., n=3. Bars with different subscripts are significantly different at P=0.05 (Tukey's procedure).


A
Percent composition
40
35
30
25
20
15
10
5
0
a
14:0
16:0
16:1
18:0
18:1
18:2
20:0
20:4
Fatty acids


B
Percent composition
40
35
30
25
20
15
10
5
0
a
14:0
16:0
16:1
18:0
18:1
18:2
20:0
20:4
Fatty acids

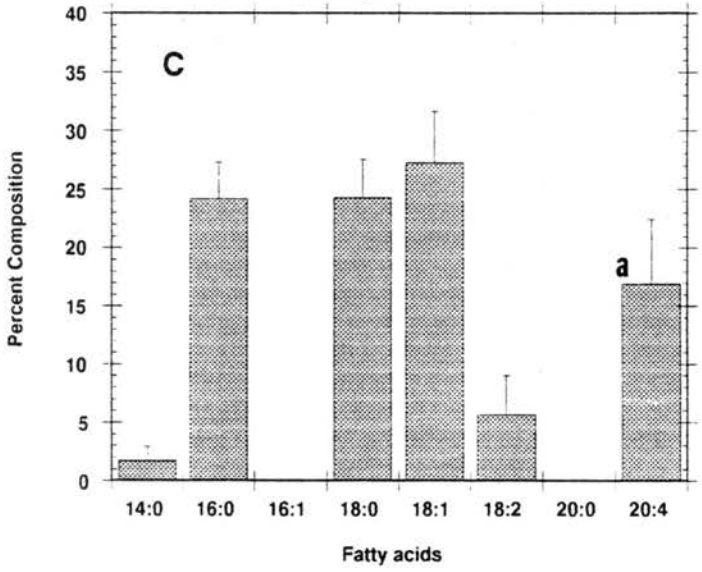
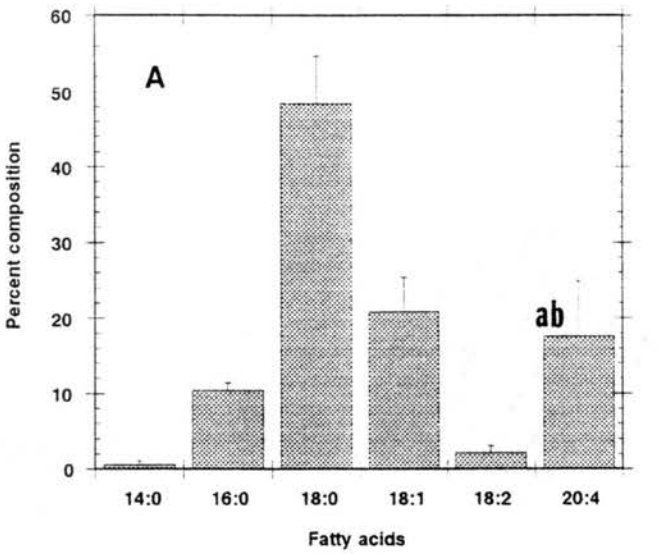
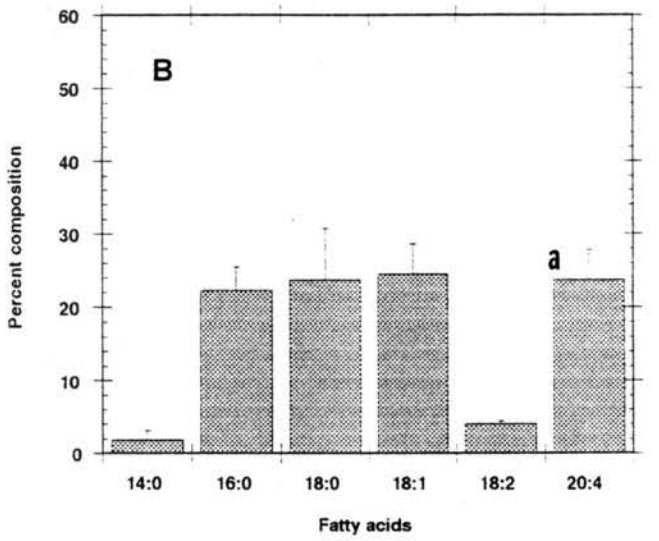

C
Percent Composition
40
35
30
25
20
15
10
5
0
a
14:0
16:0
16:1
18:0
18:1
18:2
20:0
20:4
Fatty acids

Figure 3. Fatty acid composition of (A) diacyl, (B) alkylacyl, and (C) alkenylacyl subclasses of ethanolamine phospholipids in salivary glands of slow feeding lone star ticks. Mean percent composition ± S.D., n=3. Bars with different subscripts are significantly different at P=0.05 (Tukey's procedure).


A
ab
Percent composition
60
50
40
30
20
10
0
14:0
16:0
18:0
18:1
18:2
20:4
Fatty acids


B
a
Percent composition
60
50
40
30
20
10
0
14:0
16:0
18:0
18:1
18:2
20:4
Fatty acids

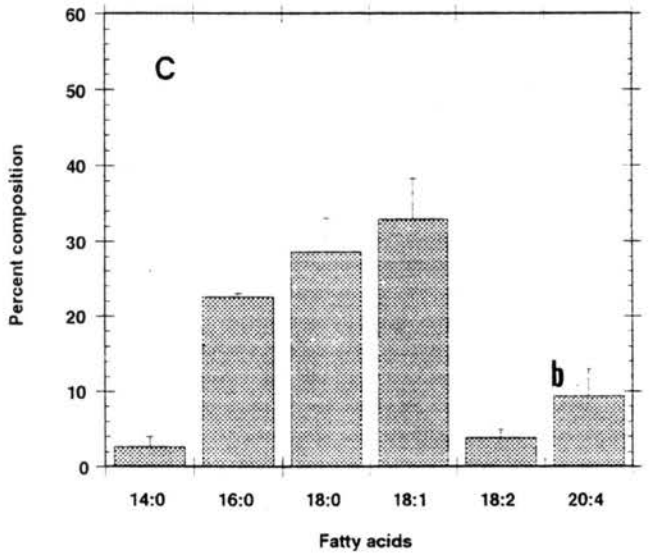

C
b
Percent composition
60
50
40
30
20
10
0
14:0
16:0
18:0
18:1
18:2
20:4
Fatty acids

Figure 4. Temporal redistribution of [^{3}H]-arachidonic acid among phosphatidylcholine and phosphatidylethanolamine after a 15 min pulse. Mean percentage ± S.D., n=3. (●) PC, (○) PE.

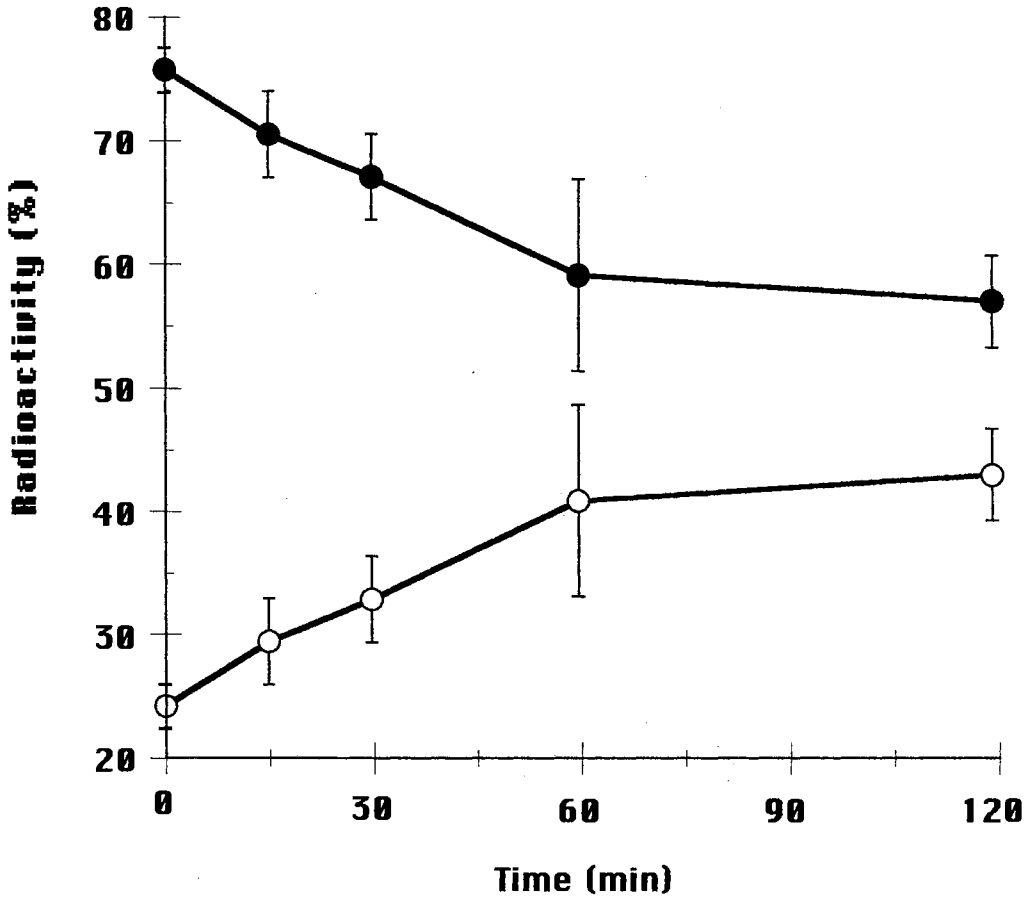

80
70
60
50
40
30
20
Radioactivity (%)
0
30
60
90
120
Time (min)

Figure 5. Temporal remodeling of [^{3}H]-arachidonic acid in diacyl-PC after a 15 min pulse. All points from three separate experiments are shown.

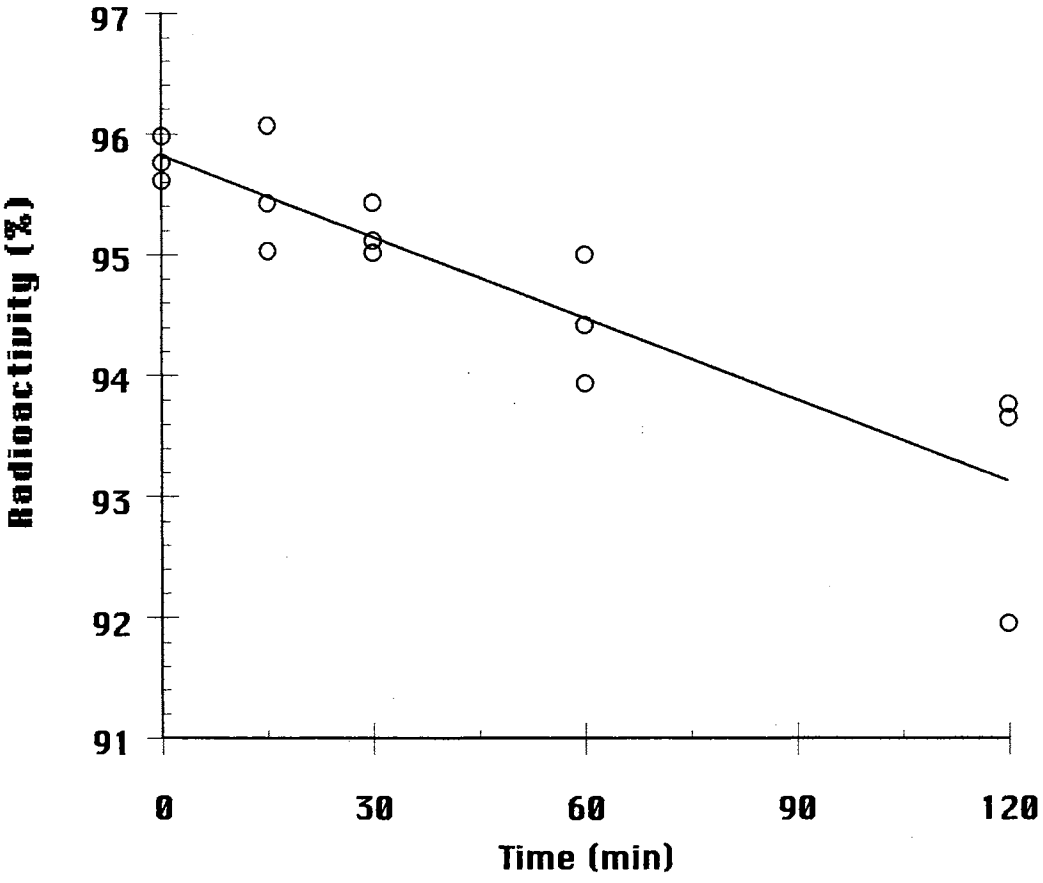

97
96
95
94
93
92
91
Radioactivity (%)
0
30
60
90
120
Time (min)

Figure 6. Temporal remodeling of [^{3}H]-arachidonic acid in (○) alkylacyl-PC and (Δ) alkenylacyl-PC after a 15 min pulse. All points from three separate experiments are shown.

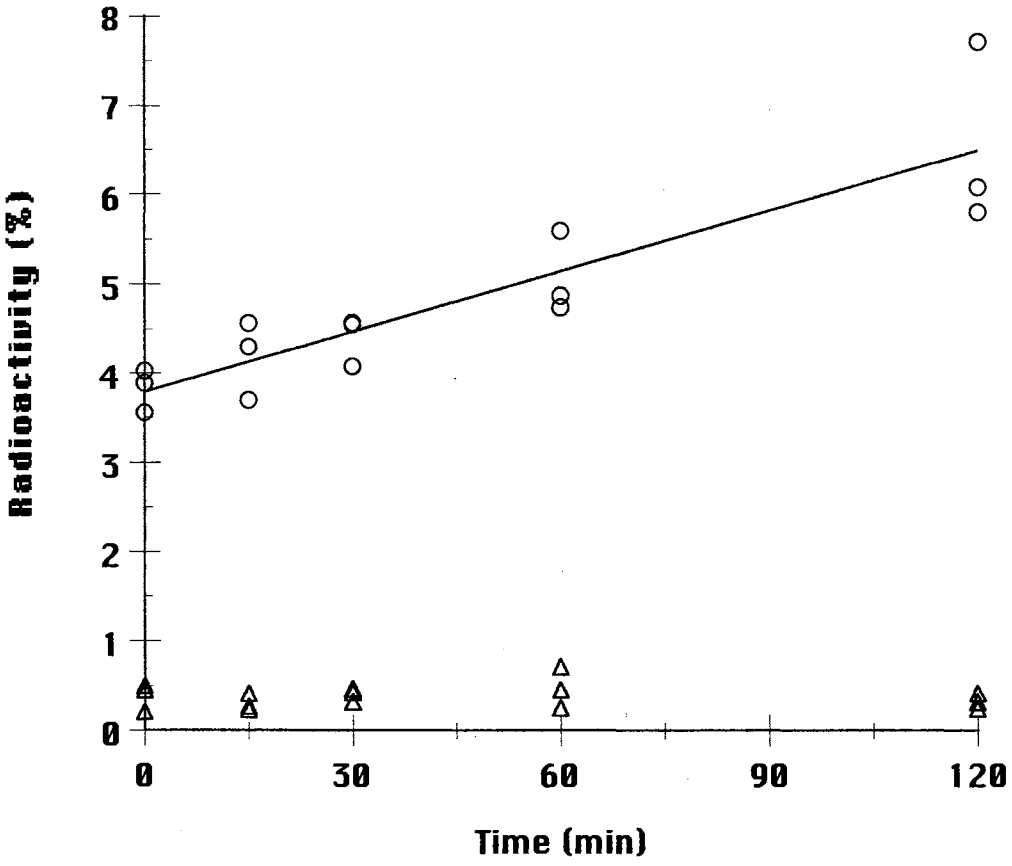

Radioactivity (%)
8
7
6
5
4
3
2
1
0
0
30
60
90
120
Time (min)

Figure 7. Temporal remodeling of [^{3}H]-arachidonic acid in diacyl-PE after a 15 min pulse. All points from three separate experiments are shown.

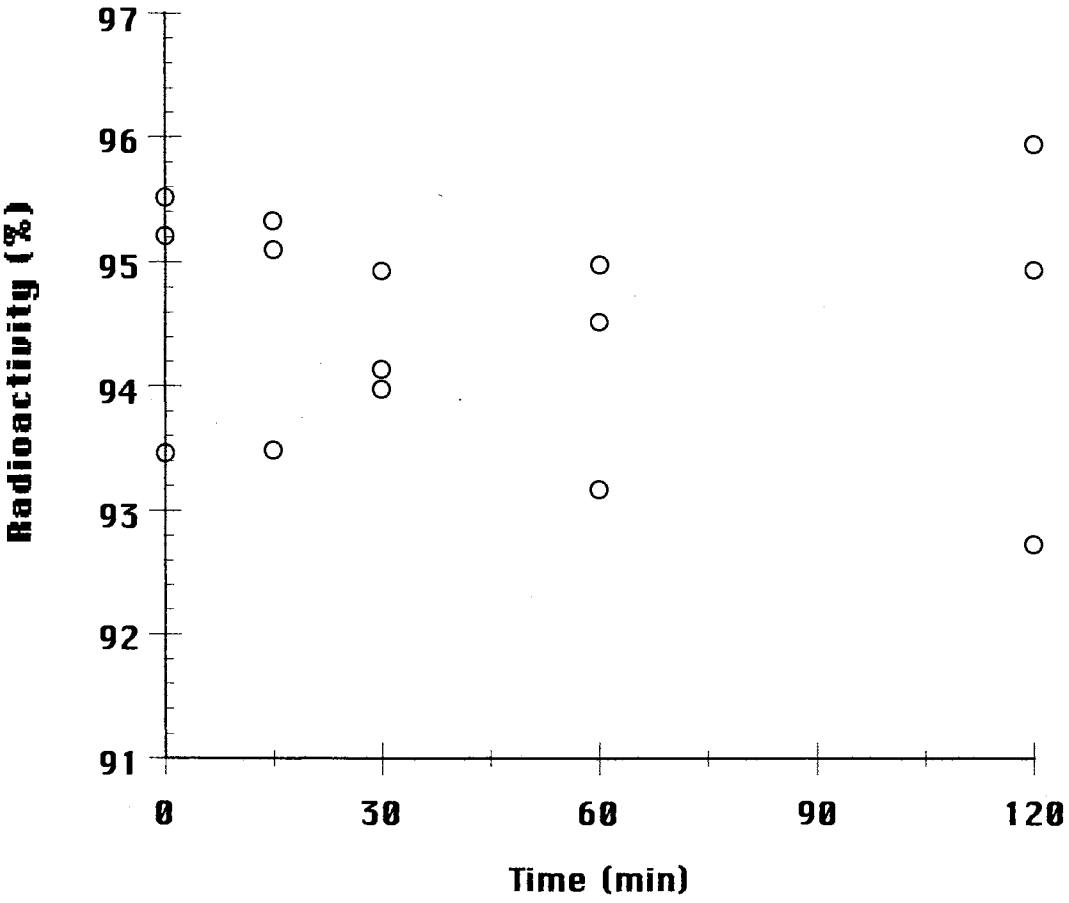
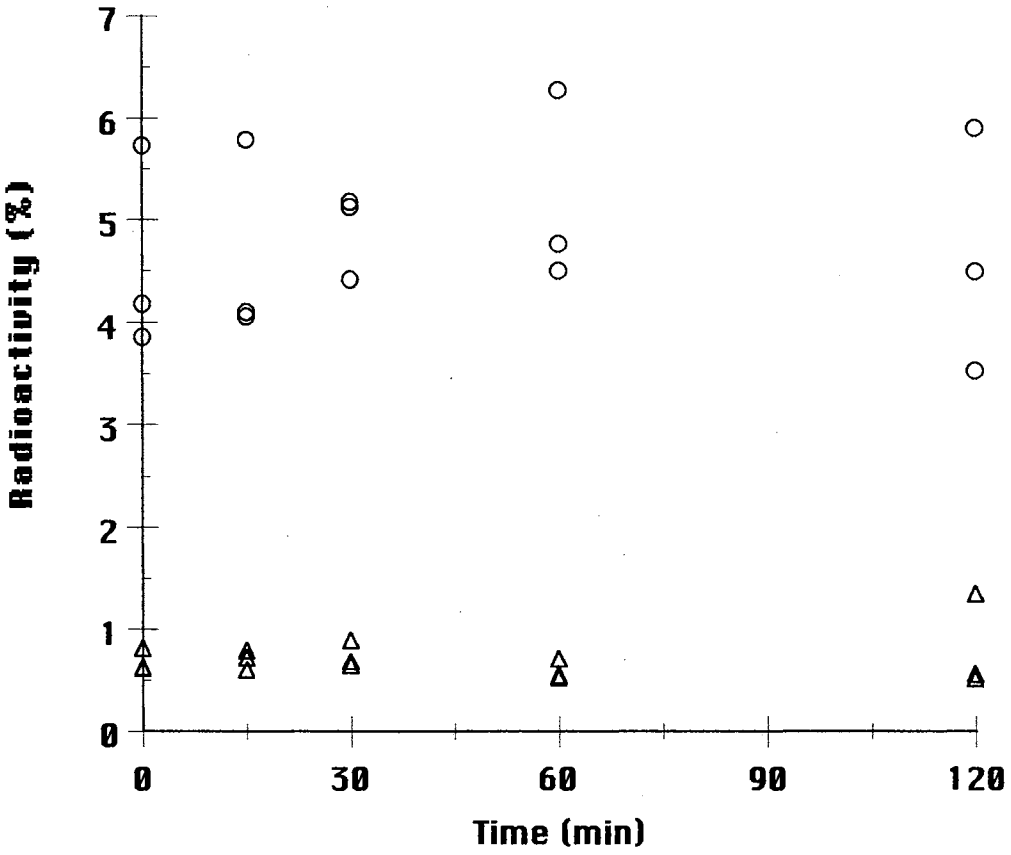

97
96
95
94
93
92
91
Radioactivity (%)
0
30
60
90
120
Time (min)

Figure 8. Temporal remodeling of [^{3}H]-arachidonic acid in (○) alkylacyl-Pe and (△) alkenylacyl-PE after a 15 min pulse. All points from three separate experiments are shown.


Radioactivity (%)
7
6
5
4
3
2
1
0
0
30
60
90
120
Time (min)

VITA

Michael McClellan Shipley

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE OCCURRENCE, DISTRIBUTION, AND REMODELING OF ARACHIDONIC ACID IN PHOSPHOLIPID FRACTIONS FROM THE SALIVARY GLANDS OF AMBLYOMMA AMERICANUM (L.)

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