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CHARACTERIZATION OF THE LIPID COMPOSITION OF WASHED AND PERCOLL GRADIENT CENTRIFUGED EPIDIDYMAL MOUSE SPERM

by

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Capacitation is an important yet poorly understood process during which the fluidity of the sperm membrane increases to prepare sperm for the acrosome reaction (AR) and subsequent sperm-egg binding. While cholesterol efflux may be partially responsible for this increase, modification of lipid components, such as remodeling of specific phospholipid (PL) bound unsaturated fatty acyl chains, may also be involved, since no change in the cholesterol:PL molar ratio following capacitation in mouse has been previously described. In this study, lipid classes (cholesterol, PL, sulfogalactosylglycerolipid (SGG), diacylglycerol and triacylglycerol) were quantified and the fatty acyl chain compositions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were characterized in washed non-capacitated (WS), washed capacitated (WCS), and Percoll gradient centrifuged (PGC) capacitated (PGCS) epididymal mouse sperm. PGC is a method that selects for the population of sperm that is highly motile, and these sperm shown to bind more sperm per egg than washed cap sperm. There were no significant differences in the levels or molar distribution of the lipid classes in WS and WCS. PGCS had significantly lower levels of cholesterol and PL compared to the washed samples, while the molar distribution of SGG was almost double that in the washed samples. The increased molar distribution of SGG, a sperm specific sulfoglycolipid, in PGCS sperm suggests its importance in fertilization. Fatty acid methyl esters were generated from PC and PE by acid methanolysis and their identity was analyzed by gas chromatography. There was a decrease of 20:4n6 in WCS and PGCS, compared to WS in both PC- (0.167 nmole/μg DNA in WS vs. 0.117 and 0.009 nmole/μg DNA in WCS and PGCS, respectively) and PE-bound (0.115 nmole/μg DNA in WS vs. 0.001 and 0.012 nmole/μg DNA in WCS and PGCS, respectively) fatty acids. These results suggest that activation of phospholipase A_2 may occur during capacitation, resulting in the release of 20:4n6, which may be further metabolized to prostaglandins that may be involved in signaling the AR. An increased distribution of PC-bound 22:5n6 and 22:6n3 in PGCS was also observed. These results also suggest that the lipid composition of PGCS, i.e., low levels of cholesterol and PL, and increased molar distribution of SGG and PUFAs, may be an important factor in achieving their increased fertilizing ability.

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LIST OF ABBREVIATIONS:

AR	Acrosome reaction
$CHCl_3$	Chloroform
DAG	Diacylglycerol
DMA	Dimethylacetal
DSC	Differential scanning calorimetry
FAME	Fatty acid methyl ester
GC	Gas chromatography
HPTLC	High performance thin layer chromatography
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
MeOH	Methanol
MS	Mass Spectrometry
MUFA	Monousaturated fatty acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGC	Percoll gradient centrifugation
PGCS	PGC capacitated epididymal mouse sperm
PI	Phosphatidylinositol
PL	Phospholipid
PLA_2	Phospholipase A_2
PLC	Phospholipase C

PM	Plasma membrane
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty ccid
SFA	Saturated fatty acid
SGC	Sulfogalactoslyceramide
SGG	Sulfogalactosylglycerolipid
SPH	Sphingomyelin
TAG	Triacylglycerol
TLC	Thin layer chromatography
WS	Washed epididymal mouse sperm
WCS	Washed capacitated epididymal mouse sperm
ZP	Zona pellucida

CHAPTER ONE

INTRODUCTION

1.1 Rationale

Comparison of the lipid composition of sperm from fertile donors with that of patients suffering from infertility has suggested that sperm fertilizing ability may be partially dependent on specific lipid components. Sperm from unexplained infertile patients show 50% lower levels of phospholipid (PL) compared to fertile donors, while cholesterol levels remain unchanged (Sugkraroek et al., 1991). This results in a cholesterol:PL molar ratio that is two times higher in the unexplained infertile patients. Analysis of the fatty acyl chain composition of sperm PL has shown that sperm from asthenozoospermic and oligozoospermic males have a lower molar distribution of 22:6n3, the major polyunsaturated fatty acid (PUFA) found in human sperm PL, compared to sperm from normozoospermic males (Zalata et al., 1998; Conquer et al., 1999). The level of this PL-bound PUFA has been positively correlated with sperm motility (Connor et al., 1997; Conquer et al., 1999), and it may also regulate normal fertilizing ability. Thus, it appears that a specific lipid composition is necessary to maintain normal sperm function.

Sperm must undergo capacitation before acquiring the ability to fertilize the egg. One of the changes associated with capacitation is an increase in membrane fluidity that has been attributed to a decrease in the cholesterol:PL molar ratio in most species. Previous work performed in our laboratory using our mouse model showed that the cholesterol:PL molar ratio of sperm before and after incubation in albumin-containing capacitating medium was the same (Tanphaichitr et al., 1996). However, using fluorescence recovery after photobleaching (FRAP) analysis, conventionally washed

mouse sperm incubated in capacitating medium showed an increase in the probe diffusion rate in the anterior region of the head, midpiece and tail when compared to non-capacitated controls (Wolf et al., 1986). These results suggest that there is an increase in membrane fluidity during capacitation and may be attributed to changes in other lipid components. Therefore, the objective of this thesis was to characterize the major lipid classes (i.e., cholesterol, PL, diacylglycerol, triacylglycerol and sulfogalactosylglycerolipid) and the distribution of PL-bound fatty acyl chains following incubation of washed mouse sperm in capacitating medium to determine whether any changes in these lipid components occurred during capacitation that may be involved in preparing the sperm for the acrosome reaction (AR) and ultimately fertilization. For example, increased membrane fluidity during capacitation in mouse sperm may result from an increased distribution in PL-bound PUFAs, while other lipids components may have different functions for fertilization. Release of the fatty acid 20:4n6, possibly resulting from phospholipase A2 activation during capacitation, may be involved in signaling the AR (Meizel and Turner, 1984). Also, generation of diacylglycerol as a result of phospholipase C activation has been shown to be involved in signaling the AR (Roldan, 1999).

Another objective was to characterize the lipid composition of sperm prepared by Percoll gradient centrifugation (PGC), which selects highly motile sperm having higher fertilizing ability. This was performed to determine how the lipid composition of PGC sperm, which may most represent those involved in fertilizing the egg, differed from those prepared by conventional washing.

1.2 Structure of the Sperm Cell

Sperm are highly polarized cells with a distinct compartmentalized structure (Nolan and Hammerstedt, 1997). The two major domains are the head and the tail, with the latter further divided into the midpiece and the principal piece (Figure 1.1A). The entire sperm is enclosed by a plasma membrane (PM). In the head region underlying the PM are the outer and inner acrosomal membranes that envelope the acrosomal contents, and the nuclear membrane. Different regions of the sperm have specific roles that are significant for fertilization. For example, the head region, which contains egg receptors, is involved in sperm-egg interactions. It also houses the male genome, which is transferred into the egg at the time of fertilization. The midpiece, having mitochondria, provides energy for sperm activities; and the tail, which contains the axoneme, is involved in propelling the sperm (Eddy, 1988; Nolan and Hammerstedt, 1997).

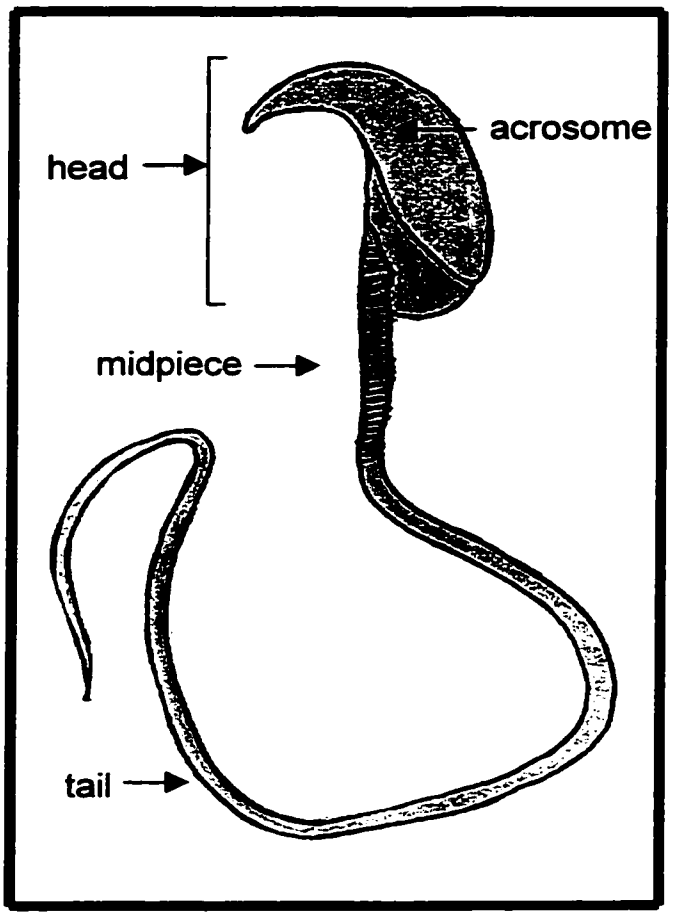
1.3 Acquisition of Sperm Fertilizing Ability: Sperm Maturation, Capacitation and Acrosome Reaction

The process of sperm production in the testes is called spermatogenesis. It includes two meiotic divisions and subsequent specialized differentiation steps (spermiogenesis) that shape the round spermatids into testicular sperm (Yanagimachi, 1994). These sperm migrate from the caput to the caudal epididymis and are stored in the caudal epididymis until ejaculation, where they mature and gain forward motility patterns (Figure 1.2). However, these sperm do not yet have the capacity for fertilization.

Sperm gain fertilizing ability only after undergoing two important physiological processes in sequence - capacitation and the acrosome reaction (AR) (Figure 1.1B). Capacitation is an important, yet poorly understood, process during which biochemical

Figure 1.1: A) Structure of a mouse sperm. The sperm head contains the acrosome, and receptors involved in binding with the egg, the midpiece contains the mitochondria, and thus provides energy for sperm activities, and the tail propels the sperm. B) Sequence of the acrosome reaction: a) cross section of a sperm head, b) fusion of the plasma membrane (PM) with the underlying outer acrosomal membrane resulting in a release of the acrosomal contents, c) and d) exposure of the inner acrosomal membrane (IAM). Ac refers to the acrosome. Adapted from Yanagimachi, 1994.

A)



B)

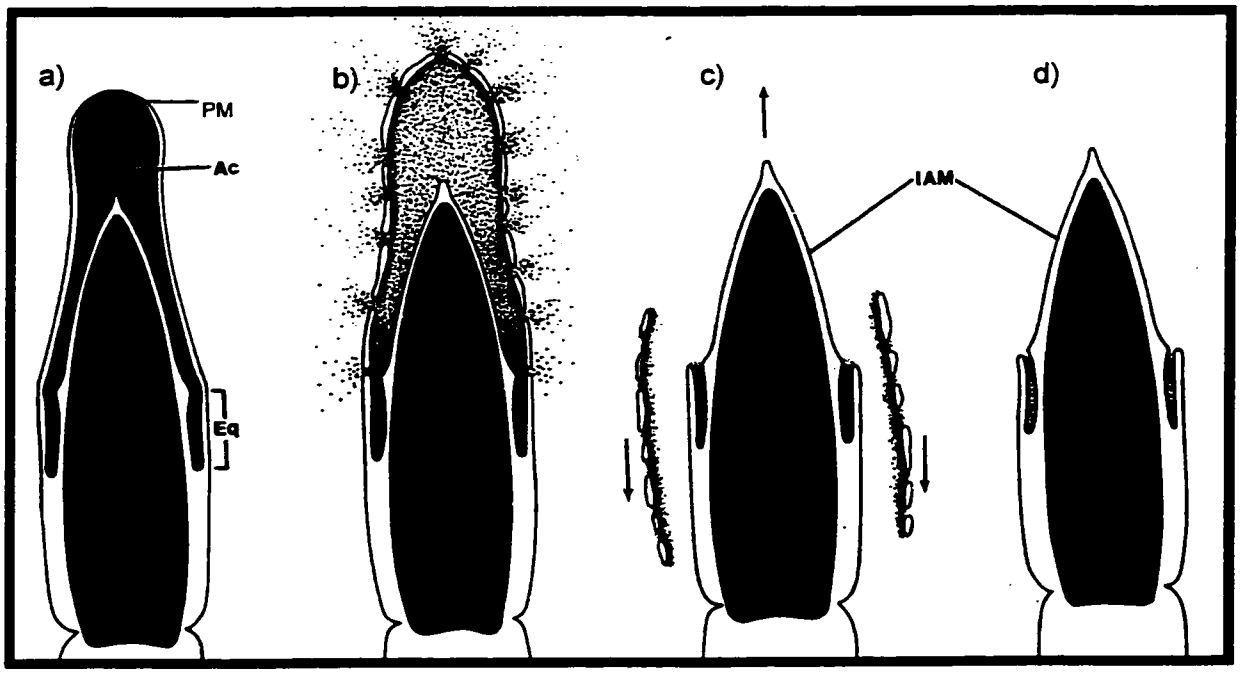
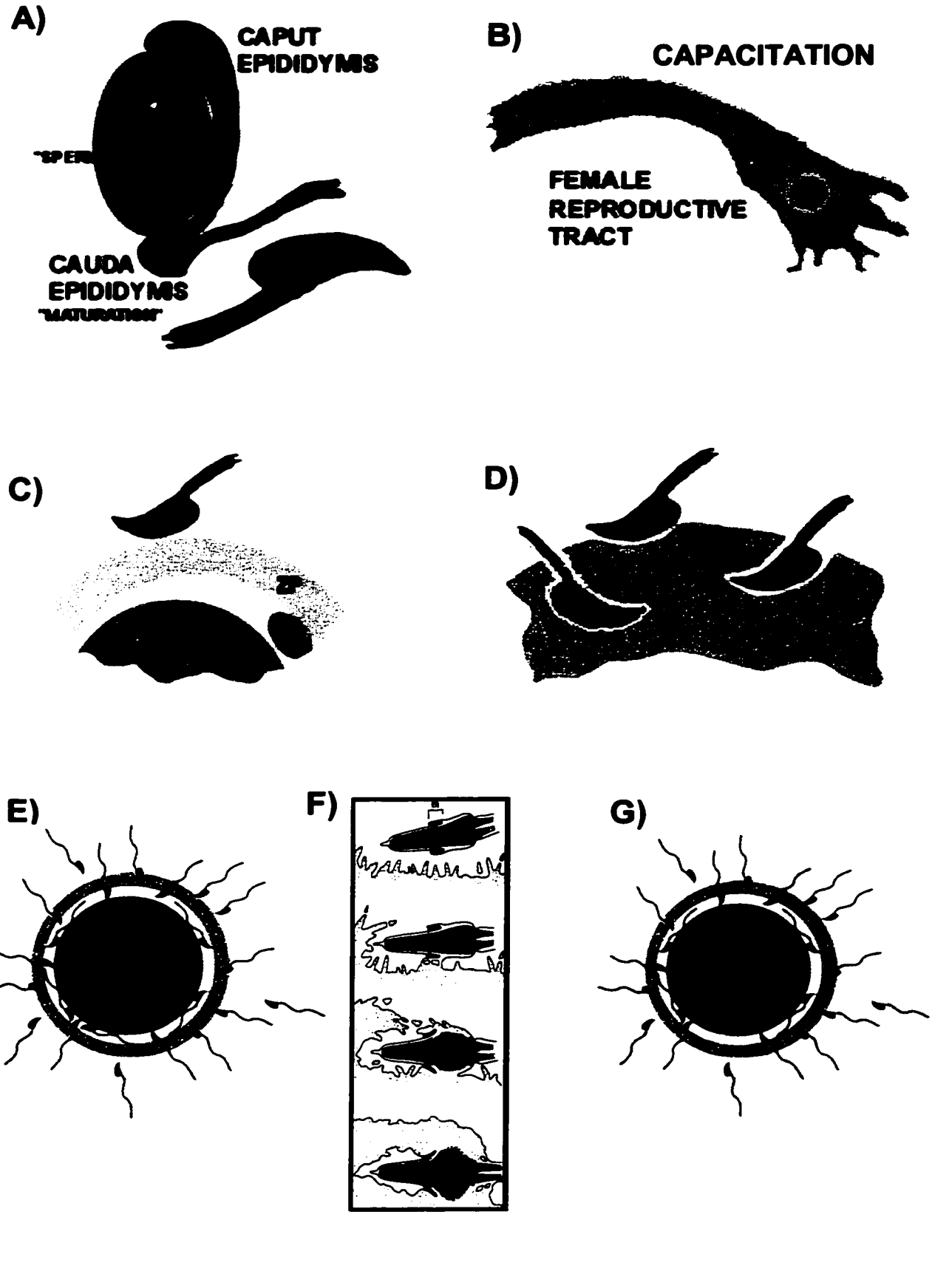


Figure 1.2: Overview of fertilization. Sperm are synthesized in the testis and undergo maturation, which involves modification of the PM and development of forward motility patterns, as they travel from the caput to the caudal epididymis. Sperm are stored in the caudal epididymis until ejaculation (A). Following ejaculation, residence in the female reproductive tract causes sperm to undergo capacitation (B), during which modifications occur including exposure of egg receptors on the sperm surface, development of hyperactive motility patterns and increased PM fluidity surrounding the acrosome. Only capacitated sperm can undergo the AR, which is initiated by binding to the ZP glycoprotein surrounding the egg (C). Many sperm undergo the AR, penetrate the ZP (D) and bind to the egg PM, which occurs initially at the apical tip of the sperm and then at the equatorial segment (E). Sperm-egg PM fusion is initiated when one acrosome reacted sperm comes into contact with the egg PM at its equatorial segment (F – taken from Yanagimachi, 1994), until ultimately, the sperm is incorporated within the egg proper resulting in sperm decondensation, which is demonstrated by swelling of the sperm head (G).


A)
CAPUT
EPIDIDYMIS
CAUDA
EPIDIDYMIS
B)
CAPACITATION
FEMALE
REPRODUCTIVE
TRACT
C)
D)
E)
F)
G)

and physiological changes occur to prepare sperm for the AR, and egg penetration (Flesch and Gadella, 2000). It occurs in vivo in the female reproductive tract, which contains albumin and high density lipoproteins that act as sterol acceptors, inducing cholesterol efflux from the sperm PM overlying the acrosome (Davis, 1981; Langlais et al., 1988). Changes during capacitation also include development of hyperactivated sperm motility patterns (Burkman et al., 1990; Martinez and Morros, 1996), exposure of egg receptors on the sperm surface (Yanagamachi, 1994), a small influx of extracellular Ca^{2+} (Ruknudin and Silver, 1990; Harrison et al., 1993; Parrish et al., 1999), and an increase in membrane fluidity (Wolf et al., 1986) resulting from a decrease in the cholesterol:PL molar ratio in most species (Iborra et al., 2000). There is also an increase in cAMP that appears to be involved in cell signaling, including protein tyrosine phosphorylation that is regulated via the action of protein kinase A (Leclerc et al., 1995; Visconti et al., 1995; de Lamirande and Gagnon, 1997).

Capacitation is followed by the acrosome reaction (AR) (Figure 1.1B). The AR is induced by binding of capacitated sperm to an egg zona pellucida (ZP) glycoprotein (i.e., ZP3 - the primary sperm receptor in mouse) (Florman and Wassarman, 1985). It is initiated by fusion of the PM of the sperm head anterior with the underlying outer acrosomal membrane, resulting in vesiculated structures and finally the release of the acrosomal contents. It is characterized by a major influx of extracellular Ca^{2+} and a release of the contents of the acrosome, containing a number of hydrolytic enzymes, which digest the ZP, thus boring a track for sperm passaging. This fusion event is made possible by the increased fluidity of the sperm membrane achieved during capacitation. The newly exposed inner acrosomal membrane of the acrosome reacted sperm bind to

ZP2, the secondary sperm receptor in mouse (Bleil et al., 1988). The acrosome reacted sperm penetrate through the ZP and bind to the egg PM, followed by the fusion of one sperm with the egg PM and subsequent incorporation of the sperm into the egg proper (Figure 1.2). This fusion causes the cortical granule reaction, in which the membranes of the egg cortical granules fuse with the egg PM, resulting in the release of the granule contents containing hydrolytic enzymes. This causes a 'hardening' of the egg ZP, thus preventing

Primary binding of sperm occurs at the ZP of the egg and many sperm surface molecules have been implicated to be involved in mediating this sperm-egg adhesion, and signaling the events involved in fertilization. ZP adhesion molecules involved in primary sperm-ZP binding must be on the surface of live acrosome intact sperm in the head region, and in mouse, they are expected to bind to ZP3. The current candidate ZP-associated molecules include: arylsulfatase-A (Tanphaichitr et al., 1999), SGG (White et al., 2000), and β 1,4-galactosyltransferase (Shur and Neely, 1988; Snell and White, 1996; Wassarman, 1999). The AR is initiated immediately after primary binding of a sperm to the ZP, and exposure of intra-acrosomal proteins that are involved in secondary sperm-ZP binding occurs, to ensure a more secure attachment of the sperm to the ZP. In mouse, the secondary sperm receptor is ZP2. Proteins involved in secondary binding include sp38 (Mori et al., 1995), proacrosin (Moreno et al., 1999; Richardson and Orand, 1996) and PH-20 (Hunnicutt et al, 1996). Once the sperm have undergone the ZP-induced AR, they further penetrate through the ZP into the perivitelline space and bind to the egg PM. Binding occurs at the equatorial segment of the sperm PM, and fusion results. Molecules that have been shown to be involved in sperm-egg PM fusion include fertilin (ADAM1,

proteins with A Disintegrin And Metalloprotease domain) (Primakoff and Myles, 2000), protein DE (Cohen et al., 2000) and SGG (Ahnonkitpanit al, 1999) on the acrosome reacted sperm head surface. Although many sperm and egg surface molecules are being targeted in the study of sperm-egg interactions, it seems likely that many molecules are involved and may work by either acting together, or as backups for one another, at the level of the ZP as well as during adhesion and fusion of the gamete PMs (Wassarman, 1999).

1.4 The Plasma Membrane

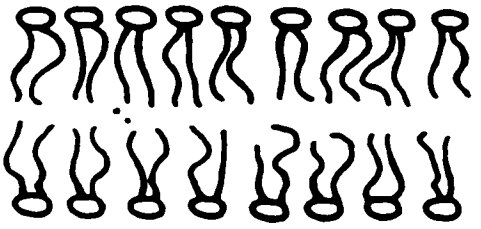
The PM is the sub-cellular component that encloses the cytoplasm and forms a barrier between the extracellular milieu and the cytoplasm, and between one cell and another. The building blocks of membranes are lipids, of which phospholipids (PL) are the major component. PLs spontaneously assemble into bilayers in a process driven by the shielding of the hydrophobic acyl chains of PLs from water, and the orientation of the hydrophilic head groups to hydrogen bond with water (Van Voorst and De Kruijff, 2000). Proteins are also present in the membrane and may be either integral to the membrane or associated with the membrane surface (Singer and Nicolson, 1972), and may be conjugated to lipids or carbohydrates (Kooyman et al., 1998). Glycoproteins or glycolipids contain sugar residues, and are located in the outer leaflet of the membrane with the sugar moieties oriented towards the extracellular space (Vos et al., 1994). Lipids exist in a wide variety of chemical structures having variation in head group and hydrocarbon chain composition, suggesting that they have a greater variability in function than just forming a hydrophobic barrier between environments (Dowhan, 1997). To

allow for proper membrane function, the membrane must be in a stable, yet fluid state at physiological temperature (Singer and Nicolson, 1972; Shinitzky, 1984). The properties of PLs contribute to the regulation of membrane fluidity as a result of the varying lengths and degree of unsaturation of the hydrocarbon chains, the interactions of the polar head groups with the external environment and adjacent PLs, and the types of chemical linkages of the hydrocarbon chains to the lipid backbone. Other major lipid classes present in the mammalian membrane include neutral lipids (e.g. sterols, diacylglycerol and triacylglycerol) and glycolipids. Together these lipids form the bilayer structure of the membrane.

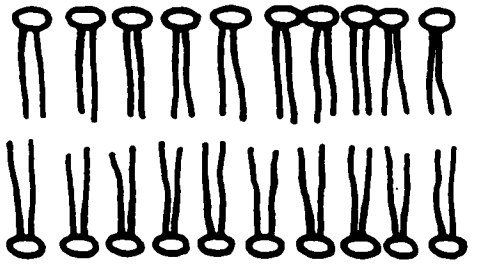
Lipid molecules in an aqueous system interact to form a variety of structures, which are classified as lamellar or non-lamellar phases (Marsh, 1991; Tate et al., 1991), and these structures depend on the lipid species and composition, and temperature (Marsh, 1991). The most biologically relevant phase is the lamellar phase, which consists of lipid bilayers separated by layers of water, such that the polar head groups are oriented towards the water (Shinitzky, 1984; Tate et al., 1991). The lamellar phase can be sub-classed according to conformation, orientation and packing of the hydrocarbon chains, as well as the interfacial hydration of these lipids. In the liquid crystalline phase (L_α), the hydrocarbon chains in the bilayer interior are highly disordered and undergo motional fluctuations, while in the gel phase (L_β) the hydrocarbon chains are rigid and planar (Shinitzky, 1984; Marsh, 1991) (Figures 1.3A and B). Another important structure is the hexagonal type II phase, in which cylinders of water are embedded in lipid, and is driven by lipids in which the polar head group is smaller than the average cross section in the hydrocarbon region (Figure 1.3C) (Shinitzky, 1984). Various techniques can be used

Figure 1.3: Illustration of A) the liquid crystalline phase, B) gel phase, and C) the hexagonal type II phase of membranes. Adapted from Shinitzky, 1984.

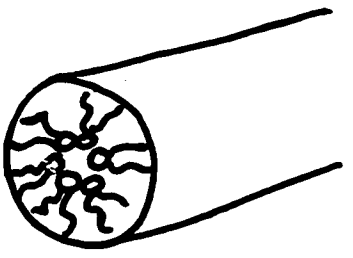
(A) Liquid Crystalline Phase



(B) Gel Phase



C) Hexagonal Type II Phase



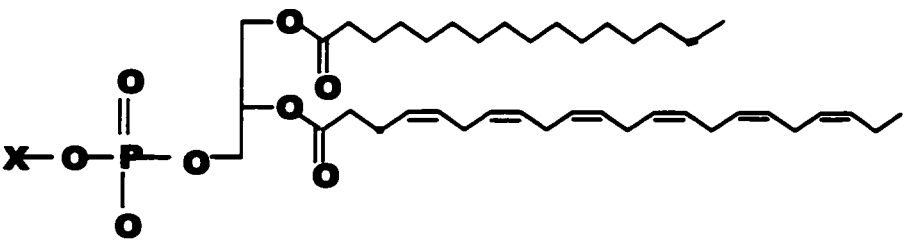
to determine the thermotropic behaviour of lipids, i.e., the behaviour of the lipids with changing temperature. Techniques such as differential scanning calorimetry (DSC) and thermotropic Fourier-transform infrared (FTIR) spectroscopy give information about the physical state of lipid bilayers in the form of a phase transition temperature (T_m), the temperature at which the membrane goes from one phase to another, e.g. from the gel phase to the liquid crystalline phase.

1.4.1 Phospholipids

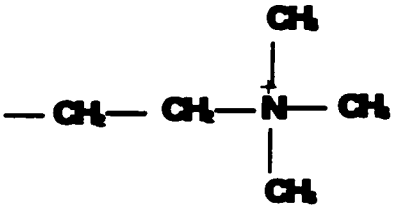
PLs can be divided into phosphoglycerolipids and sphingomyelin (SPH). The basic structure of a phosphoglycerolipid consists of two nonpolar hydrocarbon chains attached to a glycerol backbone at the *sn*-1 and *sn*-2 positions, and a phosphate esterified to the *sn*-3 position (Figure 1.4A). The phosphate is esterified to a variety of head groups. Similar to the phosphoglycerolipids, SPH has a phosphorylated head group. However, it is based on a sphingosine backbone instead of glycerol (Figure 1.4B). PLs are amphipathic molecules (i.e., having a hydrophilic head group and hydrophobic hydrocarbon chains) and their polar head groups orient towards the exterior of themembrane, and usually form hydrogen bonds with the aqueous phase, while the hydrocarbon chains form the hydrophobic interior of the membrane.

The three major PL classes in the PM are phosphatidylcholine (PC), phosphatidylethanolamine (PE) (Figure 1.4A), and sphingomyelin (SPH) (Figure 1.4B), with smaller contributions from phosphatidylserine (PS) and phosphatidylinositol (PI). PC is the most abundant PL class in the mammalian PM. It is present mainly in the outer leaflet of the bilayer and mainly plays a structural role (Van Voorst and De Kruijff, 2000). PE is present mainly in the inner leaflet of the membrane and has been suggested

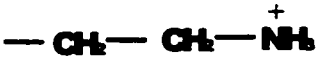
Figure 1.4: Structure of the major phospholipid classes and fatty acid chain linkages. A) The basic structure of a phosphoglycerolipid having a glycerol backbone, where X represents the head group, and the two fatty acid chains are attached via ester (acyl) linkages. B) The chemical structure of sphingomyelin, which has a ceramide backbone. C) A saturated fatty acid linked via an ether bond at the *sn*-1 position and an ester linked monounsaturated fatty acyl chain (18:1n9) at the *sn*-2 position. D) A plasmalogen, which has an alk-1-enylether linked saturated fatty acid at the *sn*-1 position and an acyl linked polyunsaturated fatty acyl chain (22:6n3) at the *sn*-2 position.



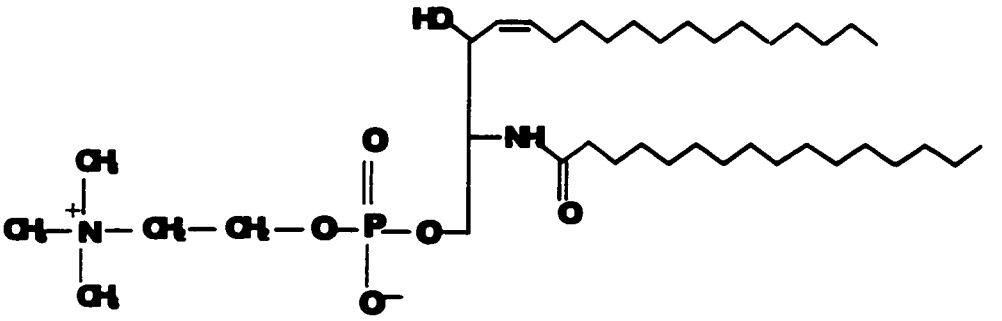
X:



phosphoglycerocholine
(phosphatidylcholine, PC)



phosphoglyceroethanolamine
(phosphatidylethanolamine, PE)



sphingomyelin

to play a role in regulating protein folding (Bogdanov and Dowhan, 1999). Thermotropic studies of PE and PC having identical fatty acyl chains showed that PC had a lower melting temperature, suggesting that PC increased membrane disorder compared to PE. This is because the primary amine of PE can form hydrogen bonds with adjacent phosphate groups, increasing the order of the membrane (Shinitzky, 1984; Boggs, 1987). Also, the head group of PC occupies more space than PE, thus providing more space for its fatty acyl chains to occupy (Shinitzky, 1984). SPH is present mainly in the outer leaflet of the membrane and contains mainly saturated fatty acyl chains, thus SPH has the ability to stabilize the membrane (Barenholz and Thompson, 1980). Metabolites of SPH, such as sphingosine-1-phosphate and ceramide, also function as second messengers regulating cell proliferation or apoptosis, respectively (Spiegel and Milstien, 1995; Levade and Jaffrezou, 1999). PS and PI, although they are present as minor components in membranes, also play significant roles. PS is translocated from the inner to the outer membrane of the bilayer during apoptosis, and thus serves as a marker for cells undergoing apoptosis (Fadok et al., 1998), while PI plays an important role in cell signaling (Dove et al., 1999; Backer, 2000).

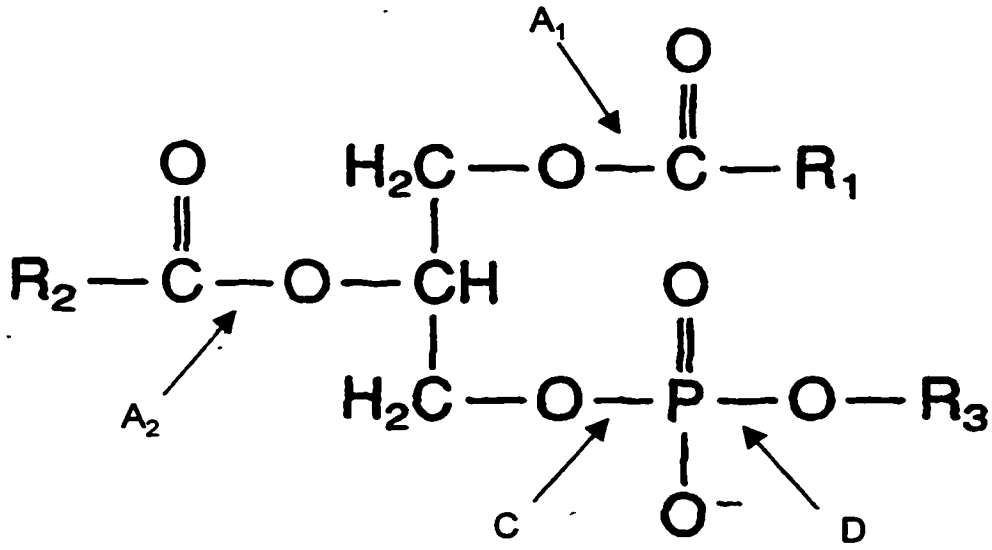
Great variety exists in the length and the degree of unsaturation of the fatty acyl chains attached to the glycerol backbone of PLs. In mammalian cells, the fatty acyl chains contain an even number of carbons and the length usually varies from 14 to 24 carbons. The chains can be saturated (SFA), monounsaturated (MUFA - containing one double bond) or polyunsaturated (PUFA - containing two or more double bonds), and the double bonds are of the *cis* type. Generally, the *sn*-1 position contains a saturated or monounsaturated fatty acid such as 16:0, 18:0 or 18:1n9, while the *sn*-2 position contains

an unsaturated fatty acid such as 18:1n9, 20:4n6 or 22:6n3 (Lands, 2000). The hydrocarbon chain can be attached at the *sn*-1 position in one of three different ways – via an acyl, alkyl or alk-1-enylether (plasmalogen) linkage, while the hydrocarbon chain at the *sn*-2 position is generally attached via an acyl linkage (Figure 1.4C and D). The presence of unsaturated fatty acids is expected to increase membrane fluidity, since the *cis* double bonds form kinks in the fatty acyl chains, and thus occupy more space in the bilayer as compared to PLs containing SFAs.

Plasmalogens (Figure 1.4D) are ubiquitously found as a constituent of mammalian cell membranes. The presence of the ether bond in PE plasmalogens has been shown to slightly increase the gel-to-liquid crystalline phase transition temperature and significantly decrease the liquid crystalline-to-hexagonal type II phase transition temperature (Paltauf, 1994). The biological function for this class of lipids is not clearly understood, but possible roles have been described. Plasmalogens are more susceptible to oxidative reactions compared to their fatty acid ester analogues due to the reactivity of their enylether function, therefore, they may play an antioxidant role (Engelmann et al., 1994; Zoeller et al., 1999). Ethanolamine plasmalogens are also a major storage depot for arachidonic acid (20:4n6 fatty acid) (Blank et al., 1973; Ford et al., 1989), which can be further metabolized to eicosanoids that play a role in inflammatory reactions (Heller et al., 1998; Reilly et al., 1998). The generated lysoplasmalogens may be involved in the biosynthesis of platelet-activating factor (PAF) (Nieto et al., 1991; Sugiura et al., 1991).

PLs are hydrolyzed by enzymes called phospholipases, which are involved in PL metabolism, membrane remodeling and signal transduction (Figure 1.5). The actions of phospholipases A_1 and A_2 result in the release of the fatty acyl chain in the *sn*-1 and *sn*-2

Figure 1.5: Sites of action of phospholipases. A_1 and A_2 represent phospholipases A_1 and A_2, respectively, C represents phospholipase C, and D represents phospholipase D.


A1
O
H2C—O—C—R1
O
R2—C—O—CH
O
H2C—O—P—O—R3
A2
C
O−
D

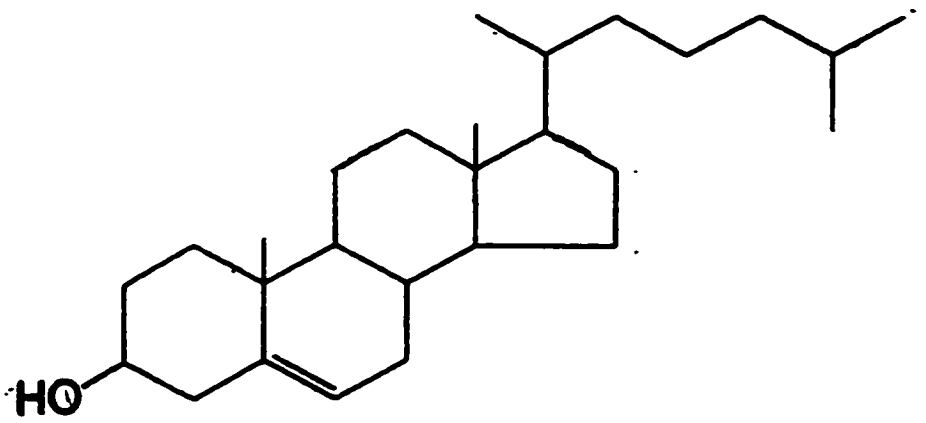
positions, respectively, yielding a lysophospholipid, a product that can destabilize the membrane and cause membrane fusion (Riffo and Parraga, 1997). Some PLAs show multiple activities including PLA_1, PLA_2, lysophospholipase, transacylase and/or fatty acyl-CoA hydrolase activity (Dennis, 1994). The transacylase activity of PLA_2 may be of special importance since it has been shown to play a role in membrane PL remodeling (Seeds and Bass, 1999; Lio and Dennis, 1998; Balsinde et al., 1995; Reynolds et al., 1993). In particular, this activity has been described to be involved in the incorporation of 20:4n6 into lysoPLs of murine $P388D_1$ macrophages (Lio and Dennis, 1998; Balisinde et al., 1995) and CHO cells (Lio and Dennis, 1998). The action of phospholipase C results in the removal of the phosphorylated head group with the generation of diacylglycerol, which plays a role in cell signaling pathways by activating protein kinase C (Ron and Kazanietz, 1999). The role of phospholipase D is to cleave the head group from the phosphorylated glycerol backbone (i.e., phosphatidic acid).

1.4.2 Neutral Lipids

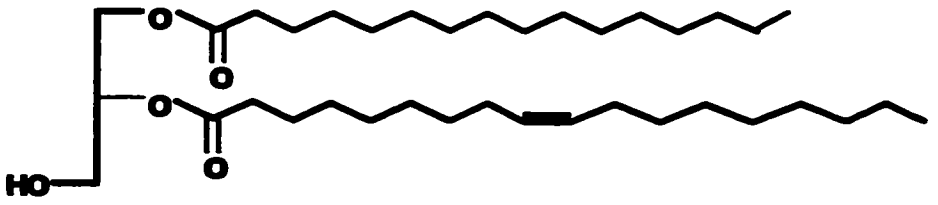
Sterols are the most common neutral lipid, with cholesterol being the most abundant sterol in mammalian cells. It has a planar multi ring structure, a polar hydroxyl group and a hydrocarbon tail (Figure 1.6A). Like the PLs, it is an amphipathic molecule with a polar hydroxyl group facing the aqueous phase and the hydrophobic steroid rings buried within the hydrocarbon chains of the PLs. Cholesterol affects the packing of membrane lipids and has a fluidizing effect on lipids in the gel state, while it tends to condense lipids in the liqud crystalline state (Tocanne et al., 1994). A preferential

Figure 1.6: Chemical structures of the major neutral lipid classes – A) cholesterol, B) diacylglycerol, and C) triacylglycerol.

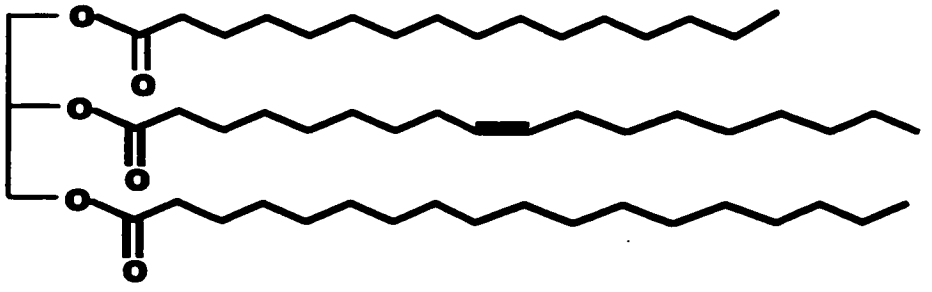
A)



B)



C)



interaction between cholesterol and sphingolipids has been demonstrated (Brown and London, 2000). This interaction may be the basis for the main component of raft membranes that have been suggested to form platforms that are involved in lateral sorting and functioning of certain proteins and during signal transduction events, and may form potential docking sites for certain pathogens and toxins (Brown, 1998; Brown and London, 2000).

Two other classes of neutral lipids are triacylglycerols (TAG) and diacylglycerols (DAG) (Figure 1.6B and C). TAG is the main energy source for biological tissues and fatty acids are usually stored and transported in the form of TAG. Cells of most tissues synthesize TAG, but only a few (e.g., adipose tissue and liver) are adapted to store TAG in any quantity (Mulder et al., 1999). In other tissues, synthesis, storage and lipolysis of TAG may be involved in regulating the production and availability of molecules such as DAG and fatty acids, which participate in signal transduction (Mulder et al., 1999), or in providing a source of fatty acids for PL synthesis (Unger et al., 1999). DAG, on the other hand, has been demonstrated to be important in cell signaling by acting as a secondmessenger. In most cells, the action of DAG is related to the activation of protein kinase C (Ron and Kazanietz, 1999).

1.4.3 Glycolipids

The general structure of glycolipids consists of various sugar molecules attached to a lipid backbone, and the backbone may be glycerol (glyceroglycolipid) or ceramide (e.g., cerebrosides. gangliosides, globosides) (Gurr and Harwood, 1991). Glycolipids play a role in cell-cell/extracellular matrix adhesion and ligand-receptor interactions via the interaction of their sugar residue(s) with binding ligands (Curatolo, 1987). A variety

of sulfated glycolipids have been described in different cell types. For example, sulfogalactosylceramide (SGC) constitutes 4 to 7 mole% of total lipids in the myelin sheath (Murray et al., 1980; Murray and Narashimhan, 1990), and is also found in testes of birds, fish, reptiles, amphibians and certain mammals including humans (Murray and Narashimhan, 1990). SGC is also a major lipid component of sea urchin sperm raft membranes (Ohta et al., 1999). Sulfogalactosylglycerolipid (SGG) is the major glycolipid in mammalian germ cells (5 to 8 mole% of total lipids) (Ishizuka, 1997) (Section 1.5). The negatively charged sulfate group of these sulfoglycolipids may play a role in cation trapping (Tupper et al., 1992, 1994) and in subsequent cation transport into cells, and since SGG and SGC are located in the outer leaflet of the PM, they are also involved in cell adhesion (Curatolo, 1987). SGC and SGG, as well as their parent glycolipids, GC and GG, have been shown to interact in vitro with gp 120, the surface glycoprotein of HIV-1 (Harouse et al., 1991) and to infertility related mycoplasmas (Lingwood et al., 1990). Sperm SGG has also been demonstrated to play a role in sperm-egg interactions (Ahnonkitpanit et al., 1999; White et al., 2000).

1.5 Membrane Lipids of Spermatogenic cells, Testicular Sperm and Epididymal Sperm

Changes in membrane lipid composition are observed throughout spermatogenesis and the lifespan of sperm, e.g., as spermatogenic cells divide and differentiate to spermatozoa, following storage and maturation of sperm in the epididymis, and then residence of mature sperm in the female reproductive tract. During the testicular phase, the spermatogenic cell membrane is fluid to allow for cell division and morphological specialization, while the membrane of sperm is more stable during epididymal storage in preparation for ejaculation and to prevent the possibility of

premature capacitation or AR. Finally, during passage through the female reproductive tract, membrane destabilization in the anterior acrosomal region of the sperm head occurs in preparation for the potential interaction between gametes, during which the AR also takes place (Nolan and Hammerstedt, 1997). These changes in membrane stability and fluidity during spermatogenesis, spermiogenesis, capacitation, and the AR are dictated by the lipid components of the male germ cell membrane.

The most abundant PL classes in the male germ cell membrane are PC, PE and SPH, with lesser amounts of PS and PI (Hall et al., 1991; Nikolopoulou et al., 1985; Parks and Hammerstedt, 1985; Rana et al., 1991). The lipid composition of male germ cells is unique since they contain a high proportion of plasmalogens (20 to 40%), as compared to somatic cells (Poulos et al., 1973; Nikolopoulou et al., 1985; Parks and Lynch, 1992; Lin et al., 1993; Brouwers et al., 1998), although the function of this PL sub-class is not clearly understood. The antioxidant property of plasmalogens (Engelmann et al., 1994; Zoeller et al., 1999) may be of significance in sperm, since they are enriched in PUFAs (Nikolopoulou et al., 1985; Aveldano et al., 1992; Brouwers et al, 1998; Lenzi et al., 2000), such as 20:4n6, 22:5n6 and 22:6n3 (Scott, 1973; Parks and Hammerstedt, 1985; Nikolopoulou et al., 1985; Aveldano et al., 1992; Hall et al., 1991; Brouwers et al., 1998; Lenzi et al., 2000). Preferential enrichment of 22:5n6 and 22:6n3 is species dependent; 22:5n6 is enriched in rat (Aveldano et al., 1992), boar (Nikolopoulou et al., 1985; Parks and Lynch, 1992), and stallion sperm (Parks and Lynch, 1992), while 22:6n3 is enriched in human (Zalata et al., 1998; Conquer et al., 1999; Ollero et al., 2000) and bull sperm (Parks and Lynch, 1992). The presence of these PUFAs may be necessary to provide the sperm membrane with the increased fluidity that

is required for PM and outer acrosomal membrane fusion during the AR, as well as subsequent fusion of the PM of acrosome reacted sperm with the egg PM.

The head region (acrosome and postacrosome) of sperm has been shown to be significantly more fluid than either the midpiece or the principal piece in human, boar and ram sperm (Wolfe et al., 1998; James et al., 1999a,b), and the existence of functional barriers to diffusion have been demonstrated (Wolf and Voglmayr, 1984; Wolf et al., 1988). In intact boar and human sperm, phase transition temperatures of 18°C and no distinct transition, respectively, were revealed using FTIR (Drobnis et al., 1993). The lack of transition temperature in human sperm was confirmed by Laurdan Fluorescence spectroscopy (Palleschi and Silvestroni, 1996) and has been attributed to the high levels of cholesterol in human sperm compared to other species (Darin-Bennett and White, 1977). Varying results have been obtained for different preparations of boar sperm using a variety of methods. In isolated boar sperm PMs, transitions centered around 32 and 6°C were obtained (Canvin and Buhr, 1989), while lipids extracted from the PM showed transitions of 24°C for PL and 36°C for glycolipids (Parks and Lynch, 1992). The presence of two transitions centered around 26 and 60°C in ram sperm PM (Wolf et al., 1990) was interpreted to be due to the coexistence of gel and liquid crystalline phases at physiological temperature. The lower of the two transitions was suggested to result from the major PL classes (i.e., PC and PE), while the transition at 60°C was postulated to be a result of the presence of glycolipids or disaturated fatty acyl chains of PLs in the sperm membrane. All of these results indicate that the sperm membrane is mainly in a liquid crystalline (i.e., fluid) state at physiological temperature. However, microdomains having varying fluidities may also exist and serve a specific purpose.

1.5.1 Changes in Lipid Composition of Spermatogenic Cells

Characterization of the lipid composition of mouse testes showed that they were mainly PC (50%) and PE (24%) (Grogan, 1981). Further fatty acyl chain characterization showed that the PC fatty acyl chains were mainly 16:0 (45%), while PUFAs constituted 25%, with 20:4n6 and 22:5n6 being the major ones. In PE, 33% of the fatty acyl chains were 16:0, whereas 35% were PUFAs, with 20:4n6 being the major one (Grogan, 1981). Further studies to elucidate the fatty acid composition of developing spermatogenic cells revealed an enrichment of 22:5n6 (6 to 12%) and 22:6n3 (4 to 8%), and a decrease in 20:4n6 (10 to 6%), as cells matured from primary spermatocytes to condensing spermatids (Grogan et al., 1981). In rats, in terms of the PL class distribution, no change in PC composition was observed (46%), while PE increased (23 to 28%), as the cells matured from spermatocytes to late spermatids (Beckman et al., 1978). An enrichment of 22:5n6 (10 to 20%) in the total PLs of the more mature cells was also observed (Beckman et al., 1978).

1.5.2 Changes in Lipid Composition During Epididymal Storage

Changes in the lipid composition during epididymal storage have been evaluated by comparing caput epididymal sperm with mature caudal epididymal sperm. During transport through the epididymis, the membrane composition of sterols and/or PL has been observed to decrease in many mammalian species (rat: Hall et al., 1991; Aveldano et al., 1992; boar: Evans and Setchell, 1979; Nikolopoulou et al., 1985; bull: Lavon et al., 1970; Quinn and White, 1967; Parks and Hammerstedt, 1985; goat: Rana et al., 1991), with PE being the PL class observed to decrease most significantly (rat: Aveldano et al.,

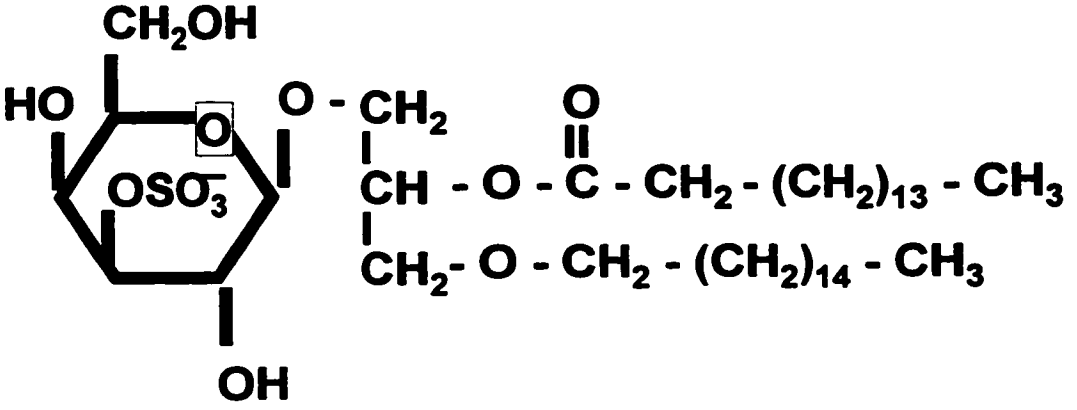
1992; boar: Nikolopoulou et al., 1985, Parks and Hammerstedt, 1985; human: Grogan et al., 1966; Poulos et al., 1973; goat: Rana et al., 1991). The change in the molar ratio of cholesterol:PL varies depending on the species: it decreases in rats (Hall et al., 1991), remains the same in boars and rams (Nikolopoulou et al., 1985; Parks and Hammerstedt, 1985) and increases in goats (Rana et al., 1991). A change in the distribution of PL-bound fatty acids has been observed, with a decrease in SFAs and an increase in unsaturated fatty acids observed in many mammalian species (Aveldano et al., 1992; Nikolopoulou et al., 1985). The opposite was observed in goat in which there was an increase in SFAs (Rana et al., 1991).

The PL composition of epididymal mouse sperm has been demonstrated to be PC (66%), PE (13%) and SPH (21%) (Alvarez et al., 1987). A decrease in the amount of 22:6n3 was observed in epididymal sperm compared to sperm collected from the seminiferous tubules of the testes (3 vs. 1 nmole per million sperm in testes and epididymes, respectively), as a result of a decrease in the PL levels, while the molar distribution of this fatty acid increased (3 vs. 6% in testicular and epididymal sperm, respectively) (Ollero et al., 2000).

1.6 Sulfogalactosylglycerolipid

Sulfogalactosylglycerolipid (SGG) (Figure 1.7), commonly known as seminolipid, is the major glycolipid of mammalian sperm (about 10 mole% of total lipids) (Ishizuka, 1997). It is also found at a much lesser extent in the white matter of the brain, where it composes 0.2 mole% of total lipids (Farooqui, 1981; Murray and Narasimhan, 1990; Ishizuka, 1997). Chemical characterization of SGG reveals its structure to be mainly 1-*O*-hexadecyl, 2-*O*-hexadecanoyl, 3-3'- sulfogalactosyl glycerol

Figure 1.7: Chemical structure of sulfogalactosylglycerolipid (SGG), the major sulfoglycolipid of mammalian sperm.


CH2OH
HO
O
O - CH2
O
CH - O - C - CH2 - (CH2)13 - CH3
OSO3
CH2 - O - CH2 - (CH2)14 - CH3
OH

(Alvarez et al., 1990). SGG is synthesized in zygotene spermatocytes and remains sulfated throughout spermatogenesis, sperm maturation, and the initial stage of in vitro sperm capacitation (Kornblatt, 1979; Lingwood, 1985; Tanphaichitr et al., 1990). SGG has been implicated to be involved in sperm-egg binding. Sperm pretreated with a polyclonal antibody monospecific to SGG lose their ability to bind to the egg ZP (White et al., 2000) and the egg PM (Ahnonkitpanit et al., 1999). Additionally, SGG liposomes bind directly to the egg ZP, while GG liposomes do not (White et al, 2000), suggesting that the negative sulfate group is of importance. In another line of work, SGG was shown to increase the rigidity of dimyristoylphosphatidylcholine model membranes (Attar et al., 2000). This suggests that SGG may also be involved in forming rigid domains, similar to SGC in raft membranes of sea urchin sperm (Ohta et al., 1999), and these domains may play a role in sperm-egg binding.

1.7 In Vitro Capacitation

In vitro fertilization, performed for research studies and for alleviating infertility problems, has been advanced by the development of in vitro capacitation methods, which involve incubating sperm washed free of seminal plasma or epididymal fluid in a defined medium whose composition mimics that of the female reproductive tract (Toyoda et al., 1971). An albumin-containing medium is conventionally used, with the role of albumin discerned as a cholesterol acceptor (Davis et al., 1979; Go and Wolf, 1985; Cross, 1998), which results in a decrease of the cholesterol:PL molar ratio and thus, an increase in membrane fluidity (Davis, 1981; Go and Wolf, 1985; Cross, 1998). Various preparations of bovine serum albumin (BSA) contain associated lipids (Davis et al., 1979; Go and

Wolf, 1985) and incubation of sperm in albumin-containing medium has been shown to decrease the levels of cholesterol by about 30 to 50%, while almost doubling the levels of PLs (Go and Wolf, 1985). Only delipidated BSA was able to decrease cholesterol levels (30%) without affecting the level of PLs (Go and Wolf, 1985).

Contamination of BSA by other serum components, including enzymes, hormones, lipids, and organic and inorganic ions, has made it difficult to clearly define the molecular mechanisms of BSA-induced changes during capacitation (Choi and Toyoda, 1998). More recently, methyl-β-cyclodextrin has been used in place of albumin to induce capacitation and result in fertilization (Choi and Toyoda, 1998; Visconti et al., 1999; Iborra et al., 2000). Cyclodextrins are cyclic oligosaccharides, consisting of 6, 7 or 8 glucopyranose units, and are able to incorporate hydrophobic compounds within their core. β-Cyclodextrins (6 glucose units) have been shown to specifically induce cholesterol efflux from cultured cells (Irie et al., 1992; Ohtani et al., 1989), while minimizing the removal of PLs (Kilsdonk et al., 1995). α-Cyclodextrins (7 glucose units) specifically induce PL efflux (Irie et al., 1992; Ohtani et al., 1989) and thus, the specificity of β-cyclodextrins for cholesterol appears to be a function of the structure of the hydrophobic core (Kilsdonk et al., 1995; Irie et al., 1992).

1.8 Percoll Gradient Centrifugation

Epididymal/ejaculated sperm are heterogeneous in morphology and motility. Therefore, sperm prepared by conventional washing (i.e., centrifugation to separate seminal/epididymal fluid from collected cells) contain all populations of cells including motile sperm with normal morphology and immotile sperm with abnormal morphology.

Experiments performed using conventionally washed sperm may not represent the physiological activity of sperm having the highest fertilizing ability (i.e., motile sperm with normal morphology). A population of motile sperm having normal morphology can be prepared by subjecting ejaculated or epididymal sperm to discontinuous Percoll gradient centrifugation (PGC) (Lessley and Garner, 1983; Dravland and Mortimer, 1985; Tanphaichitr et al., 1988; Gravance et al., 1998; Lenzi et al., 2000).

Percoll solution (Pharmacia) consists of sodium-stabilized colloidal silica having particle diameters between 10 and 30 nm. The particles are coated with polyvinylpyrrolidone (PVP) to protect cells from the toxic effects of silica (Pertoft, 2000). A discontinuous gradient of 45/47% and 90% Percoll is typically used for sperm separation (Tanphaichitr et al., 1988; Tanphaichitr et al., 1996; Zalata et al., 1998). Either epididymal fluid or semen containing sperm is layered on top of the gradient and centrifugation (600 g) results in sedimentation of two sperm fractions according to their size or buoyant density, and motility. The motile sperm sediment as a pellet due to their higher density compared to immotile sperm, which possess swollen cytoplasms and incompletely condensed chromatin, and settle at the gradient interface (Tanphaichitr et al., 1988). PGC sperm have a greater fertilizing ability than sperm prepared by conventional washing (Tanphaichitr et al., 1988; Serafini et al., 1990; Grant et al., 1994; Yue et al., 1995), and they tend to possess hyperactivated motility patterns, which are characteristic of capacitated sperm, suggesting that they may be partially capacitated (De Maistre et al., 1996). Upon resuspension in BSA-containing capacitating medium, the motile PGC sperm become further capacitated (Yanagimachi 1994) and have been widely used for in vitro fertilization and artificial insemination. A disadvantage of Percoll is that

it may be contaminated with endotoxin (Pertoft, 2000) and therefore, Pharmacia, the company producing Percoll, has prohibited the use of Percoll in preparing human sperm for insemination and human IVF procedures.

1.9 Changes in Sperm Membrane Fluidity During Capacitation

The major lipid change occurring during in vitro capacitation is the removal of cholesterol from the sperm PM. Cholesterol is known to regulate the fluidity of lipid bilayers and the permeability of membranes, and modulates the lateral mobility of integral proteins and functional receptors within the membrane (Yeagle, 1991). Since cholesterol tends to have a condensing effect on lipids in the liquid crystalline phase (Tocanne et al., 1994), this decrease of cholesterol levels during capacitation causes a destabilization of the sperm PM, resulting from a decreased cholesterol:PL molar ratio and thus increased membrane fluidity (Davis, 1981; Go and Wolf, 1983). This increased membrane fluidity promotes fusion of the PM with the underlying outer acrosomal membrane during the AR (Bearer and Friend, 1982) and fusion of an acrosome reacted sperm with the egg PM (Yanagimachi, 1994). In conventionally washed human sperm induced to undergo capacitation in albumin-containing capacitating medium, it was shown that a decrease in the cholesterol:PL molar ratio was required for enhanced sperm-egg binding (Gamzu et al., 1997), suggesting that a removal of cholesterol from the sperm membrane is involved in fertilization. Other lipid changes may also be involved in increasing membrane fluidity, since the fatty acyl chain characterization of PGC human sperm, which may be capacitated, has shown an enrichment of PUFAs compared to

whole sperm (10.5 vs. 5.5% 20:4n6 and 34.5 vs. 22.5% 22:6n3 in PGC and whole sperm, respectively) (Lenzi et al., 1996).

1.10 Hypothesis

We hypothesized that changes in lipid components, such as the PL-bound fatty acyl chain distribution, might be involved in increasing membrane fluidity during capacitation. More specifically, increased distribution of PUFAs such as 22:5n6 and 22:6n3 may occur during capacitation.

1.11 Research Objectives

The first objective of this thesis was to quantify the levels of the major lipid classes of washed non-capacitated and washed capacitated epididymal mouse sperm, and PGC capacitated epididymal mouse sperm using various analytical lipid assays. These lipid classes include cholesterol, PL, SGG, DAG and TAG. Since previous work performed in our laboratory showed that there was no change in the cholesterol:PL molar ratio following capacitation (Tanphaichitr et al, 1996), we postulated that a change in the fatty acyl chain composition may be responsible for increasing membrane fluidity. Therefore, the second objective involves characterizing the fatty acyl chain composition of the major PL classes, PC and PE, of washed non-capacitated and washed capacitated epididymal mouse sperm, and PGC capacitated epididymal mouse sperm by gas chromatography (GC).

The mouse system was chosen for our lipid study because it has been the most widely used for fertilization research. While a great deal of information is known about

mouse sperm physiology and biochemistry, sperm-egg binding, and egg physiology and biochemistry (Yanagimachi, 1994), little is known about sperm lipid composition in this species. To date, the majority of the sperm lipid work has been performed mainly on large animals because of the abundance of material that is available. Unfortunately, since the number of sperm per mouse is limited, a very large number of mice are required to obtain enough lipid material to perform lipid analyses. Yet, realizing the significance of the results obtained, this study was devised to gain insight in to the process of mouse sperm capacitation The information obtained from this study can be applied to improve mouse in vitro fertilization procedures, as well as protocols for sperm cryopreservation and may improve methods for shipment and storage of mouse sperm of the best breed to be used for transgenic mouse production.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Percoll was purchased from Pharmacia (Upssala, Sweden). PC (bovine brain), PE (bovine brain), SPH (bovine brain), PS (bovine brain), and PI (porcine liver) were from Doosan Serdary Research Laboratories (Englewood Cliffs, New Jersey). DAG (16:0/16:0), TAG (16:0/18:0/16:0), cholesterol and SGC were from Sigma. Grain fatty acid methyl ester mixture was purchased from Supelco. Silica gel thin-layer chromatography plates (K6 silica gel 60 Å, 10 μm particle size, 250 μm thickness, 20 x 20 cm) and high performance thin layer chromatography plates (K6 silica gel 60 Å, 5 μm particle size, 200 μm thickness, 10 x 10 cm) were obtained from Whatman (Kent, England).

2.2 Preparation of Mouse Sperm

2.2.1 Media Preparation

The media used for sperm preparation were KRB-HEPES (119.4 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM Mg_2SO_4, 4 mM $NaHCO_3$, 21 mM HEPES, 1 mM sodium pyruvate, 5.6 mM glucose, 28 μM phenol red, pH 7.4) and KRB (same components as KRB-HEPES except that 21 mM HEPES and 4 mM $NaHCO_3$ were replaced by 25 mM $NaHCO_3$). Media were filtered through 0.22 μm filters (Millipore) into 15 ml Falcon tubes and stored at 4°C until use. KRB-HEPES and KRB supplemented with 0.3% BSA were also used and were prepared by adding the appropriate amount of Fraction V BSA (Sigma) to KRB-HEPES or KRB. The media were pre-warmed in an incubator (37°C, 5% CO_2) overnight before use. The caps of

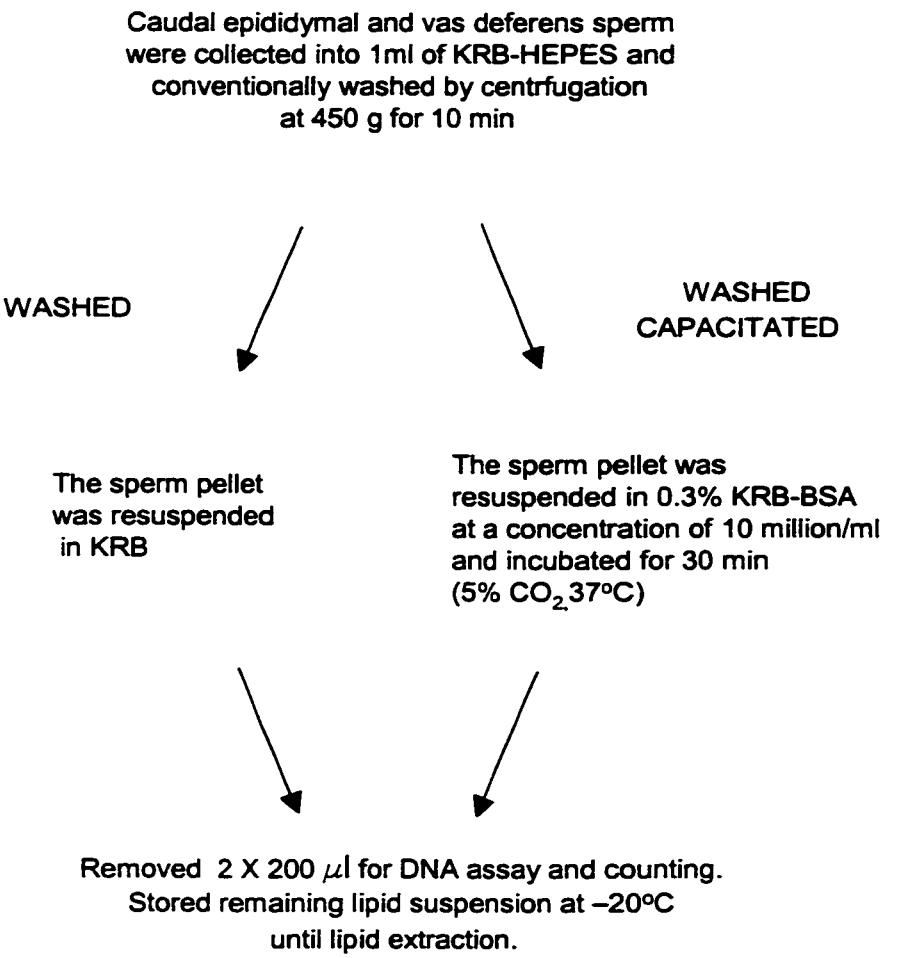
media containing HEPES were left tight during warming in an incubator (37°C), while media containing $NaHCO_3$ as the buffering system, i.e., KRB and KRB-0.3% BSA, were loosened during warming in an incubator to equilibrate with 5% CO_2 and to generate the desired pH of 7.4.

Percoll (Pharmacia) was prepared as a stock solution of 90% in KRB-HEPES, filtered through sterile 0.22 μm filters (Millipore) into 15 ml Falcon tubes and stored at 4°C until use. A batch of Percoll was obtained which tested positive for endotoxin contamination, and preparation of sperm with this batch resulted in little or no yield of sperm having poor motility. To eliminate this contamination, 30 ml of Percoll solution was dialyzed against about 800 ml 0.9% NaCl for 8 hours with three changes of the saline solution. It was then filtered through 0.22 μm filters and stored at 4°C until use. On the day of sperm preparation, the Percoll was diluted to 90% with KRB-HEPES. Percoll was allowed to equilibrate to room temperature before use.

2.2.2 Washed Non-Capacitated and Washed Capacitated Epididymal Mouse Sperm Preparation

Male CD-1 mice (10 to 12 weeks old) were fasted overnight and sacrificed on the following day by cervical dislocation. Sperm were collected from the caudal epididymis and vas deferens into 1 ml of KRB-HEPES at 37°C, with precaution to ensure removal of fat pads surrounding the tissues, since contamination by lipids in the fat tissue could alter the sperm lipid results. In the case of washed sperm (WS), the collected epididymal sperm were centrifuged at 430 *g* for 10 min at 25°C (Figure 2.1). The supernatant was removed and the sperm pellet was washed once by resuspending in KRB-HEPES and

Figure 2.1: Flow chart of the preparation of washed and washed capacitated epididymal mouse sperm.


Caudal epididymal and vas deferens sperm were collected into 1ml of KRB-HEPES and conventionally washed by centrfugation at 450 g for 10 min
WASHED
WASHED CAPACITATED
The sperm pellet was resuspended in KRB
The sperm pellet was resuspended in 0.3% KRB-BSA at a concentration of 10 million/ml and incubated for 30 min (5% CO_2,37°C)
Removed 2 X 200 μl for DNA assay and counting. Stored remaining lipid suspension at –20°C until lipid extraction.

centrifuging for 10 min at 430 *g* and 25°C. The final pellet was resuspended in KRB for lipid extraction. Washed capacitated sperm (WCS) were prepared by incubating the resuspended sperm pellet following the first centrifugation in KRB-0.3% BSA (30 minutes, 37°C, 5% CO_2) at a concentration of about 10 million sperm/ml (Figure 2.1). Following the incubation, the sperm were centrifuged for 10 min at 430 *g*, the BSA-containing supernatant was removed and the sperm pellet was resuspended to 5 to 10 million sperm/ml in KRB for lipid extraction.

The final volume of the sperm suspension was dependent upon the total number of sperm and the final concentration ranged from 5 to 10 million sperm/ml. From the final sperm suspension, 200 μl was removed in duplicate for counting and the DNA assay. From the 200 μl aliquot, 50 μl was removed and diluted 5x in KRB for counting and the remaining 150 μl was used for the DNA assay (Section 2.5). The remainder of the sperm suspension was stored at -20°C for subsequent lipid extraction and analysis.

The number of sperm required for each assay is described in Appendix A.1.

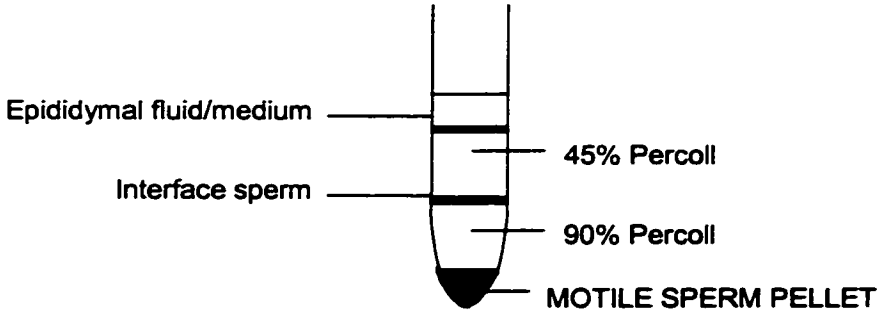
2.2.3 PGC Capacitated Epididymal Mouse Sperm Preparation

To prepare PGC capacitated sperm (PGCS), caudal epididymal and vas deferens fluid, containing sperm, was collected in 1 ml of KRB-HEPES and diluted to 2 ml with KRB-HEPES. The diluted sperm fluid was gently mixed once using a Pipetman and then loaded onto a two-step discontinuous Percoll gradient (a bottom layer of 2 ml 90% Percoll in KRB-HEPES and an upper layer of 2 ml 45% Percoll, prepared by diluting 1 ml 90% Percoll with 1 ml KRB-HEPES) (Figure 2.2). The gradient was centrifuged at 600 *g* for 30 min at 25°C. Following centrifugation, immotile sperm sedimented at the interface of the 45% and 90% layers were removed first with a transfer pipet (Fisher)

Figure 2.2: Flow chart of the preparation of PGC capacitated epididymal mouse sperm

Caudal epididymal and vas deferens sperm were collected into 1ml of KRB-HEPES and diluted to 2 ml with KRB-HEPES

The diluted sperm were layered on a Percoll gradient and centrifuged at 650 g for 30 min (25°C).



The Percoll layers were removed and the remaining motile sperm pellet was resuspended in 0.3% KRB-BSA to a concentration of 10 million/ml and incubated for 30 min (5% CO_2, 37°C)

The capacitated sperm suspension was centrifuged at 430 g for 10 min and the pellet was resuspended in KRB

Removed 2 X 200 μl for DNA assay and counting. Stored remaining lipid suspension at –20°C until lipid extraction.

without disrupting the motile sperm pellet, and then the 45% and 90% Percoll layers were removed (Figure 2.3).

The remaining motile sperm pellet (about 200 μl) was resuspended in KRB-HEPES and centrifuged at 430 *g* for 10 minutes to wash away any remaining Percoll. To capacitate sperm, the pellet was resuspended and incubated in KRB-0.3% BSA (37°C, 5% CO_2). After 30 min, the capacitated sperm were centrifuged at 430 *g* for 10 minutes to pellet the sperm and remove BSA, and the pellet was resuspended in KRB for lipid extraction. The final volume of the sperm suspension was dependent upon the total number of sperm and the final concentration ranged from 5 to 10 million sperm/ml. Aliquots were removed for DNA analysis (Section 2.5) and counting as described for washed sperm in Section 2.2.2.

The number of sperm required for each assay is described in Appendix A.1.

2.3 Sperm Counting

Aliquots for counting were removed as soon as the sperm pellet was resuspended in medium, usually within a couple of minutes, so that the effect of sperm sticking to the tube surface was minimized. Sperm sticking resulted in a cell number that was lower than the actual amount of sperm present. Sperm were diluted 5 times in either KRB or KRB-HEPES (i.e., 50 μl sperm suspension in 200 μl medium) for cell counting. A 10 μl aliquot was injected into a Bright Line haemocytometer with an improved Neubauer ruling pattern (Hausser Scientific), and having a cell depth of 0.100 mm and a total volume of 0.1 μl. The number of sperm was counted in five large squares, i.e., one square was 1mm by 1mm (Figure 2.4), under a Nikon inverted microscope at 100x

Figure 2.3: Scheme of the removal of the Percoll layer following PGC. The interface layer was removed first (A), followed by the 45 and 90% layer (B). About 200 μl of 90% Percoll, containing the motile sperm pellet, remained in the Falcon tube (C).

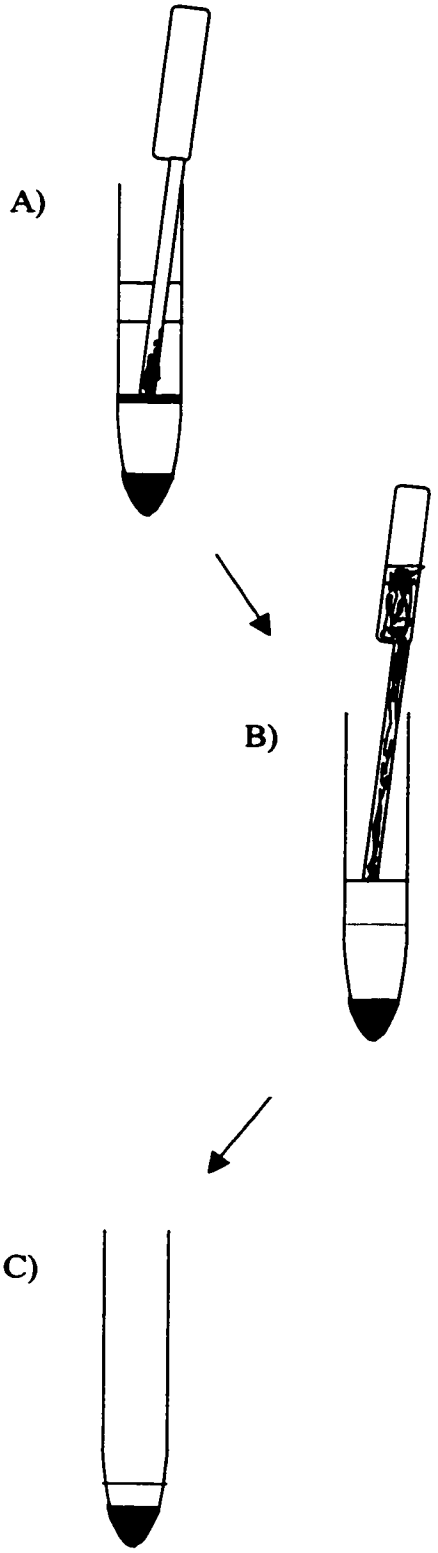
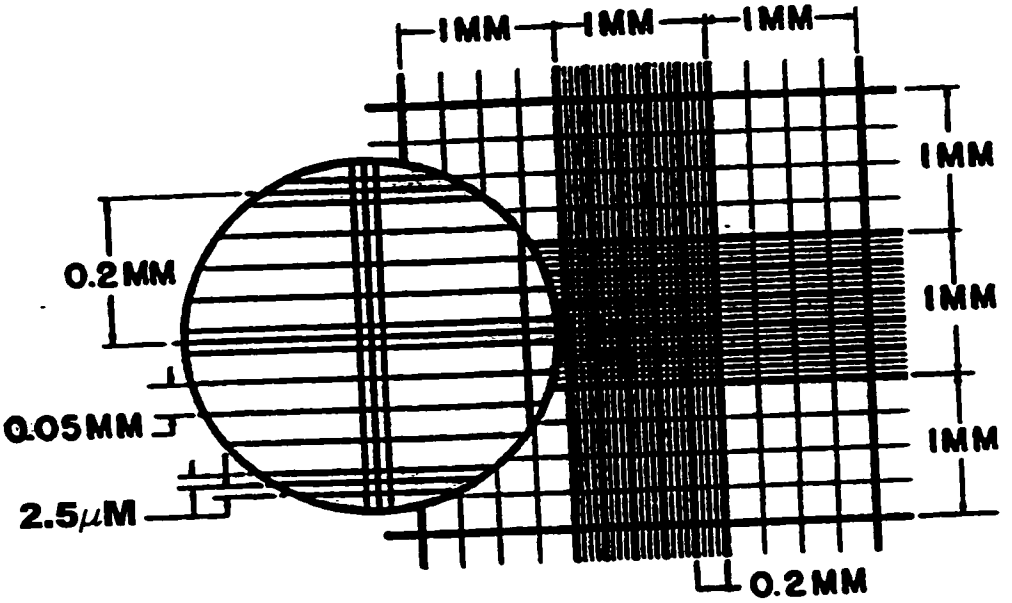

A)
B)
C)

Figure 2.4: Illustration of the Bright-Line haemocytometer. Sperm were counted in the four corner squares (all 16 squares in the 1 mm x 1 mm squares were counted), and in the center square.

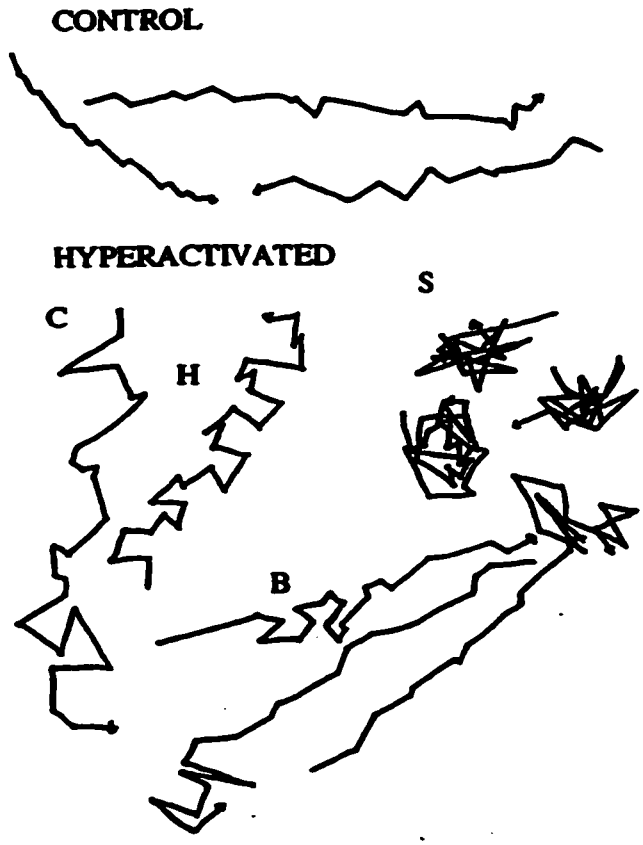

1MM
1MM
1MM
1MM
1MM
1MM
0.2MM
0.05MM
2.5μM
0.2MM

resolution. For the most accurate counting, the number of sperm had to be within a range of about 50 to 150 sperm per 1 mm by 1 mm square. The concentration was calculated using the following equation: # sperm in 5 squares x 2000 x 5 (dilution factor) = million sperm/ml. Sperm motility was assessed visually and determination of capacitation was based on the high frequency of flagellar movements and hyper-activated motility patterns (Figure 2.5).

2.4 Sperm-Egg Binding

CF1 female mice were kept in a room on a light-dark cycle of light from 1 pm to 3 am and dark from 3 am to 1 pm. Three days prior to experimentation, four females were injected with 5 to 10 IU of PMSG peritoneally at about 6 pm. The day before the experiment, the females were injected with 5 to 10 IU of hCG at about 6 pm. On the day of experimentation, the female mice were killed by cervical dislocation and the oviducts were collected. The cumulus masses were carefully removed from the oviducts by holding the oviducts with forceps and teasing with the needle of a 25 G syringe under a stereomicroscope. The cumulus masses were transferred with a >300 μm bore pipet to a pre-warmed petri dish (37°C) containing 0.1% hyaluronidase in KRB-0.3% BSA. The eggs were removed from the hyaluronidase-containing droplet with a 140 to 180 μm bore pipet and washed successively in droplets of KRB-0.3% BSA (placed in another petri dish) to remove the enzyme. The eggs were stored in a drop of KRB-0.3% BSA in the incubator until use for the binding assay, i.e., within 1 to 1.5 hours. The eggs had to be used within 16 hours post hCG injection. After this time, the eggs began to deteriorate.

Figure 2.5: Illustration of hyper-activated motility patterns observed in capacitated sperm. Adapted from de Lamirande et al., 1997. Hyper-activated motility patterns included circling high-curvature (C), helical (H) and star spin (S) patterns. Sperm hyper-activation was not constant and spermatozoa tended to change their motility patterns (biphasic, B). In general, hyper-activativation was characterized by increased frequency of movement, increased amplitude of lateral head displacement, and decreased linearity of path movement.


CONTROL
HYPERACTIVATED
S
C
H
B

WCS and PGCS were prepared as described above. Following capacitation, the sperm were diluted with KRB-0.3% BSA to give 1 million/ml and a 60 μl drop (containing about 60, 000 sperm) was placed in a petri dish. Twenty to twenty-five eggs in 1 to 2 μl of KRB-0.3% BSA were added to the drop and the gametes were co-incubated for 30 minutes in an incubator (37°C, 5% CO_2). Following incubation, loosely bound sperm were washed away and the sperm-egg complexes were transferred to the wells of a pre-warmed (37°C) 12-well glass plate into a 10 μl drop of KRB-0.3% BSA, such that there were five to ten complexes per well. The drops were covered with mineral oil (to slow the movement of the sperm-egg complexes caused by the sperm whose flagella were still beating, and to prevent evaporation of the drop) on a heating block (37°C), avoiding the generation of bubbles. The sperm-egg complexes were located and the number of sperm bound to the eggs was counted using the 10x objective lens of a Nikon TMS inverted microscope to locate the eggs, and then the 20x lens to count the sperm.

2.5 Sperm DNA Assay

Sperm DNA was assayed according to the modified method of Labarca and Paigen (1980) as previously described (Tanphaichitr et al., 1996), and was based on the binding of Hoechst 33258 dye to DNA of live sperm cells (Appendix - Figure A.1). Addition of dithiothreitol (DTT) to the sperm suspension was necessary to decondense the highly compacted sperm chromatin by disrupting protamine disulfide bonds, and to minimize the adherence of sperm cells to the surface of the assay tubes, which occurred over a short period of time, i.e., within 10 min.

The 150 μl aliquot removed from the total sperm suspension, containing about 1 million sperm, was centrifuged at 430 g for about 5 minutes, and the sperm pellet was resuspended in 100 μl of KRB-HEPES. Sperm were pretreated with 100 μl DTT (10 μmole) in KRB-HEPES for 30 minutes at 37°C. These sperm were then incubated with Hoechst-PSB (Hoechst: 1 mg/ml in Milli-Q water, stored in 50 μl aliquots at –20°C and diluted 1,000 times in PSB for use; PSB: 2 M NaCl, 0.01 M NaH_2PO_4, pH 7.4). High concentrations of salt fully decondensed the chromatin, which allowed the Hoechst dye to complex with DNA, and the intensity of the bound dye was measured using a Perkin-Elmer LS-5 Luminescence spectrometer at excitation and emission wavelengths of 360 and 458 nm, respectively.

Calf thymus DNA (Sigma) was used to construct a standard curve. The standard was prepared by cutting 5 to 10 mg of calf thymus fibrous DNA into small pieces and dissolving the pieces in 5 to 10 ml of water, with the aid of sonication. The DNA solution was dialyzed against 0.1 mM Tris-HCl (pH 7.4) overnight and then against PSB for 8 hours. Following dialysis, the DNA solution was diluted 40x with PSB and the ratio of the absorbance at 260 and 280 nm was measured. A value higher than 1.5 indicated a pure DNA solution, however, a ratio lower than 1.5 indicated contamination, and the solution had to be further dialyzed against PSB until the ratio was higher than 1.5. Assuming that double stranded DNA has a concentration of 50 μg/ml when the absorbance at 260 nm is 1 (Sambrook et al., 1989), the concentration of the dialyzed DNA was calculated, adjusted to 1 mg/ml with PSB and stored in 150 μl aliquots at –20°C. On the day of analysis, a working solution was prepared by diluting the DNA aliquot 20 times in PSB and a standard curve up to 10 μg DNA was constructed.

2.6 Lipid Extraction

Lipids were extracted from all samples following the modified method of Bligh and Dyer (1959) (Kates, 1986) (Appendix - Figure A.2). Methanol and chloroform were added to the sperm cells in KRB to give a ratio of 2:1:0.8 (methanol:chloroform:aqueous solution) and gently inverted to give a single phase. Equal amounts of chloroform and water were added to give a ratio of 1:1:0.9 (methanol:chloroform:aqueous solution) and gently inverted. This resulted in the formation of a two-phase system consisting of a lower chloroform (organic) phase, and an upper methanol plus water (aqueous) phase. Following centrifugation at 600 *g* for 2 min, the lower chloroform phase was collected and the remaining upper phase (methanol-water-protein plaque) was washed with additional chloroform of the same volume used in the original two-phase separation described above. This two-phase system was again centrifuged at 600 *g* for 2 minutes and the lower chloroform phase was collected and pooled with the first lower phase. Benzene (about 1 ml) was added to the organic phase to accelerate the evaporation of contaminating water and the sample was concentrated under a stream of N_2 at 40°C.

It was best to use glass-stoppered centrifuge tubes for lipid extractions, since Teflon-lined screw capped tubes could leak, resulting in loss of lipids. Distillation of organic solvents used for lipid extraction and handling was important since solvents could contain a small amount of contamination that could interfere with lipid work (Kates, 1986). The organic solvents used for this work were glass distilled before use by heating to their boiling points (Kates, 1986). The boiling points are as follows: chloroform, 61 to 62°C; methanol, 64.7°C; benzene, 80.1°C; petroleum ether, 54°C (Merck Index, 1983).

2.7 Glassware Preparation

To minimize contamination in the lipid assays, all glassware used for the lipid extractions and assays were pre-washed with 2% RBS®-35 Detergent Concentrated solution (Pierce, Rockford, IL). The cleaning procedure included soaking glassware overnight in a diluted detergent solution (made by adding 10 ml concentrated RBS to 1 liter of water at 50°C), followed by rinsing thoroughly 5 times with distilled water and then 3 times with Milli-Q water. The tubes for most assays were re-used and washed using this method following each use. Assay tubes were disposed of following one use in the phosphorus assay since ashing residue remained on the sides of the tubes and could not be removed by washing.

2.8 Phospholipid Assay

Phospholipid was quantitated according to the method of Duck-Chong (1979) as previously described (Tanphaichitr et al., 1996) and was based on phospholipid digestion with $Mg(NO_3)_2$ to inorganic phosphorus at high temperature (ashing) followed by a colour reaction with molybdate and malachite green (Appendix - Figure A.3). Aliquots of sperm lipid equivalent to about 0.5 million sperm were transferred in triplicate to 12 x 75 mm disposable borosilicate assay tubes (VWR, catalogue no. 60825-467) containing 10 μl of 10% $Mg(NO_3)_2$ in methanol. The lipids were dried under N_2 and ashed in the blue portion of a Bunsen flame for 30 seconds. It was important that ashing was complete and was demonstrated by the presence of a white residue on the inside surface of the assay tubes. After allowing the tubes to cool to room temperature (about 5 to 10 minutes), 300 μl of 1 M HCl was added to each tube and they were heated at 90 to 95°C

for 15 minutes, while being covered with marbles so that loss of solution due to evaporation would not occur. Following cooling to room temperature, 10 μl of 1% Triton X-100 and 600 μl of blue color reagent (4.2% ammonium molybdate (Sigma) in 4.5 M HCl and 0.3% malachite green (Sigma) in Milli-Q water in a ratio of 1:3) was added to each tube and vortexed. The product, which ranged from light brown to greenish-blue in color depending on the concentration, was measured at 650 nm using an Ultrospec 2000 UV/Visible Spectrophotometer (Pharmacia Biotech). The amount of inorganic phosphorus was quantified from a standard curve of KH_2PO_4 (0 to 0.1 μg) that was treated in the same way as the samples.

A standard curve using PC was constructed to test the reliability of PL hydrolysis, and to determine if there was any interference of the hydrolysed/ashed PL products in the assay and whether the time selected for ashing was long enough to ensure complete ashing of organic material.

2.9 Cholesterol Assay

Cholesterol was assayed employing the fluorometric method of Gamble et al. (1978), as previously described (Tanphaichitr et al., 1996), using cholesterol oxidase, peroxidase, and the substrate *p*-hydroxyphenylacetic acid (Appendix - Figures A.4 and A.5), with some modifications to reduce the amount of material required. Aliquots of sperm lipids in $CHCl_3$ were transferred to assay tubes, dried under N_2 and resuspended in MeOH. Aliquots of 10 μl of total lipids in MeOH, containing lipids extracted from 0.2 to 0.5 million sperm, were transferred to a black 96 well plate (Corning) in triplicate. A reagent solution (consisting of 4 ml 0.1 M potassium phosphate buffer, pH 7.4, 1 ml

cholesterol oxidase (1 U/ml), 1 ml peroxidase (10 U/ml), 0.5 ml 0.5% Triton X-100 in 0.1 M potassium phosphate buffer, 0.5 ml sodium cholate (8.6 mg/ml in potassium phosphate buffer), and 1.5 ml *p*-OH phenylacetic acid (6 mg/ml in potassium phosphate buffer)) was added to each well containing either standards or samples. The plate was placed at 37°C for 30 minutes and then at room temperature for 15 minutes. The stable fluorescent reaction product was measured at excitation and emission wavelengths of 325 and 415 nm, respectively, using the SpectraMAX GeminiXS (Molecular Devices). Samples were quantified from a standard curve of cholesterol (0 to 0.5 μg). Each sample was assayed in triplicate in the same 96-well plate.

2.10 Sulfolipid Assay

SGG, the only sulfolipid in mouse sperm, was quantified according to the method of Kean (1968) and was based on the formation of a colored complex between the cationic dye Azure A (Sigma) to sulfolipids, which are extractable in chloroform (Appendix - Figure A.6). An aliquot of sperm lipids equivalent to that extracted from about 20 million sperm was transferred to a screw capped tube and the solvent was removed by drying under N_2. To each tube, 1.5 ml of $CHCl_3$:MeOH (1:1, v/v), 1.5 ml of 0.05 M H_2SO_4 (in Milli-Q water) and 0.3 ml of Azure A blue dye solution (prepared by dissolving 4 mg Azure A in 0.5 ml of 0.05 N H_2SO_4 adjusted to 10 ml with Milli-Q water; the solution could be stored in a dark bottle at room temperature and was stable for about one week) was added. Each tube was vortexed for 30 seconds and centrifuged at 300 *g* for 10 minutes. The result was an upper aqueous phase that was dark blue, since it contained excess Azure A solution, and a lower chloroform phase having a faint blue

color, which reflected the presence of the Azure A-sulfolipid complex. The lower chloroform phase was carefully collected with a Pasteur pipet, transferred to a 0.5 mL quartz cuvette and the absorbance was read at 635 nm using an Ultrospec 2000 UV/Visible Spectrophotometer (Pharmacia Biotech). A standard curve of sulfogalactosylceramide (SGC) (0 to 5 μg) was constructed by subjecting known amounts of SGC to the same assay protocol as the lipid samples.

2.11 High Performance Thin Layer Chromatography (HPTLC) and Densitometric Analysis of Mouse Sperm Lipids

The major PL classes were separated by HPTLC using a solvent system of $CHCl_3$:MeOH:acetic acid:water (50:37.5:3.5:2, v/v/v/v) (Holub and Watson, 1996). The plate was pre-washed twice in the same direction, by ascending TLC, with $CHCl_3$:MeOH (1:1, v/v) and activated at 100°C for one hour. Total lipids in chloroform (about 50 μg) were spotted as a 0.5 cm band on the plate, along with authentic PL standards, and developed by ascending chromatography in a glass TLC tank lined with filter paper and pre-equilibrated with the running solvent for at least 30 minutes. Following chromatography, the plate was dried in air and then sprayed with orcinol (Sigma) - sulfuric acid solution (prepared by dissolving 100 mg of orcinol in 50 ml of 75% H_2SO_4). Orcinol is a specific stain for glycolipids, while the acid solution allows visualization of all lipids following charring (Christie, 1982). The plate was placed in an oven set at 100°C, until the lipids were visualized (about 5 to 10 minutes); glycolipids stained blue, cholesterol stained red and all other lipids stained blackish/brown.

The plate was further stained with Coomassie Brilliant Blue (Nakamura and Handa, 1984), which reacts non-specifically with all lipids. Either Coomassie blue G-250 or R-250 can be used, with Coomassie Brilliant Blue G-250 (Bio-Rad) being used in this study. The HPTLC plate was placed in 200 ml of 0.03% Coomassie blue in 30% MeOH/100 mM NaCl for 30 minutes with gentle agitation. Following staining, the plate was placed in 200 ml of destaining solution consisting of 30% MeOH/100 mM NaCl for 5 minutes with gentle agitation. When destaining was completed, the plate was removed and gently blotted with filter paper to remove excess liquid. The lipid spots stained blue in color. A photo of the HPTLC plate was taken and stored in the computer using a Biorad Gel Doc 1000 system and saved as a black and white image file (.img).

The densities of the major PL classes of each sample were analyzed using the Molecular Analyst software (Biorad). Circles were drawn around the lipid spots of interest (i.e., PC, PE and SPH) in each lane, which contained the separated lipids from one sample, and the number of pixels within the circles were counted. The % distribution of each PL class was determined based on the number of pixels per circle, in relation to the total number of pixels of all selected spots identified in one lane.

The neutral lipids DAG and TAG were separated by HPTLC using a solvent system of petroleum ether:ethyl ether:acetic acid (80:20:1, v/v/v) (Kates, 1986) and detected by Coomassie blue staining (Nakamura and Handa, 1984). They were then densitometrically analyzed, similar to what is described above. In cases where lipid spots migrated as diffuse spots, rather than concentrated bands, the plate was not used for analysis. A sample containing lipids extracted from about 5 million sperm was used for analysis. DAG and TAG standards were prepared as solutions of 250 μg/ml in

chloroform and known amounts of authentic DAG and TAG standards (0.25 to 5 μg) were spotted on each HPTLC plate using a 25 μl glass pipet. Standard curves of DAG and TAG were co-chromatographed on each HPTLC plate along with the sperm lipids. Following Coomassie blue staining, a photo of the HPTLC plate was taken and stored in the computer as a black and white image file (.img) using a Biorad Gel Doc 1000 system. Using the Molecular Analyst software (Biorad), boxes were drawn around the spots of the known amounts of DAG and TAG, along with the spots of the three samples in the same row, and the number of pixels in each box was counted. It was possible to adjust the tilt of the box to accommodate spots that may have migrated on an angle. With this software, it was possible to assign a quantity (i.e., 0.25 to 5 μg) to each known amount of DAG and TAG, and standard curves (μg DAG/TAG vs. counts) were automatically plotted. The quantity of DAG or TAG in each sample spot was determined according to the standard curve constructed using the known amounts of DAG and TAG co-chromatographed on the same plate.

2.12 FAME and DMA Analysis by GLC and GC-MS

Extracted lipids were sent to the Lipid Analytical Laboratories at the University of Guelph (Guelph, ON, Canada), for fatty acid analysis by gas chromatography (GC) under the supervision of Dr. Bruce Holub. To ensure that enough lipid material would be available, sperm from about 10 to 15 mice was required to perform this analysis for the washed samples (approximately 400 million sperm), while about 80 mice (approximately 400 million sperm) were required to analyse the PGC sperm. A known amount of unnatural di17:0 PC (Sigma) was added to the lipid sample as an internal standard and

total lipids were separated into the individual PL classes by thin layer chromatography using a solvent system of $CHCl_3$:MeOH:acetic acid:water (50:24:4:2, v/v/v/v). The separated lipids were visualized by spraying with 8-anilo-1-naphthalenesulfonic acid (ANS) (Sigma) and the PL classes of interest, i.e., PC, PE and SPH were scraped off the TLC plate into individual screw-capped test tubes. To methylate the fatty acyl chains, 2.0 ml of boron trichloride (Sigma) in methanol was added to the tubes, and was used as a trans-esterification catalyst (Christie, 1982). The tubes were tightly capped and incubated in a boiling water bath for 15 minutes. After the tubes cooled, 5.0 ml of water was added and the tubes were vortexed. To extract the fatty acid methyl esters (FAMEs), 2.0 ml of hexane was added, the tubes were vortexed and the upper hexane layer was removed into GC vials for injection. Separation of FAMEs and quantification based on the addition of a known amount of unnatural 17:0 fatty acid as internal standard, was performed using a Varian 3800 GC with a 60 meter DB-23 capillary column having a 0.32 mm internal diameter with 0.1 μm film thickness. Hydrogen was used as the carrier gas, with a flow rate of 70 cm/second. The initial injector temperature was 60°C, and the initial column temperature was 60°C and heated to 160°C at 20°C/minute and then to 200°C at 4°C/minute. The detector temperature was 250°C. The position of each FAME was determined by comparison with the retention times of authentic FAME standards, and quantification was based on comparison with the internal standard and expressed as a molar distribution.

An unknown peak, which did not correspond with any of the standards, was collected following GC analysis and sent to the laboratory of Dr. Tom Brenna at Cornell University for MS-MS analysis by Anthony Michaud.

Dimethylacetals (DMAs) and washed capacitated PE FAMEs were analyzed at Health Canada (Ottawa, ON, Canada) in collaboration with Dr. Renaud Vincent, by Dr. Premkumari Kumarathasan. To prepare DMAs and FAMEs for GC-MS analysis, the major PL classes were separated using a solvent system of $CHCl_3$:MeOH:acetic acid:H_2O (50:37.5:3.5:2, v/v/v/v) (Holub and Watson, 1996). Following chromatography, the lipids were visualized by spraying the plate with 0.01% Rhodamine 6-G solution in distilled water and viewed under ultraviolet light (Kates, 1986). PC and PE were scraped off the plate into Teflon-lined screw capped tubes and 2 ml $CHCl_3$:MeOH (1:1) was added to elute the lipids from the silica. The silica was washed twice with 2 ml volumes of $CHCl_3$:MeOH (1:1) and the pooled fractions were dried under N_2. The lipids were re-extracted by the method of Bligh and Dyer and the lower lipid-containing phase was removed and dried under N_2. To generate DMAs and FAMEs, 4.5 ml of 0.6 N methanolic HCl (0.5 ml concentrated HCl in 100 ml MeOH) was added to the dried lipids and heated at 85°C for one and a half hours. Following acid methanolysis, the tubes were allowed to cool to room temperature and 0.5 ml of water was added. The DMAs and FAMEs were extracted with three 5 ml portions of petroleum ether and the pooled extracts were concentrated under N_2. To ensure complete removal of water from the sample, it was run through a column of calcium chloride (Sigma). The column was prepared by blocking the opening of a Pasteur pipet with silanized glass wool and then filling the pipet with anhydrous sodium sulfate that had been activated above 100°C for 2 hours until about 3 cm of sodium sulfate had been added. The sample was then applied to the column and allowed to flow into a test tube placed under the column. The column

was washed three times with about 1 ml of petroleum ether, and the sample was dried under N_2 and resuspended in toluene for GC-MS analysis.

GC-MS analysis was carried out using a Varian 3400 GC equipped with an autosampler (CTC Model A-2005) and a Finnigan MAT Magnum ion trap detector (operated using the Magnun software system 2.40 for PC). Separation was performed using a 25 metre DB-5 column (0.25 mm internal diameter, 0.1 μm thickness) and helium was used as the carrier gas, at a flow rate of 28.57 $cm.s^{-1}$. The GC injection port, transfer line and the detector manifold temperatures were 270°C, 280°C, and 300°C, respectively. The column temperature was initially held at 80°C for 1 minute, then increased at a rate of 10°C/minute to 300°C where it was held for 20 minutes. Ionization was in the electron impact (EI) mode with an emission current of 10 μA, and an electron multiplier voltage of 1650 V. The mass range scanned was 40-650 amu with the automatic gain control setting, and the total ion spectrum was obtained. The scan rate was 1 sec^{-1}. Automated injections (1 μl) of liquid sample were made on this GC.

DMA standards were prepared from palmitaldehyde (sodium bisulfite) and stearic aldehyde (sodium bisulfite) (K and K Laboratories, Inc., New York). The lipids, in powdered form, were dissolved in 4.5 ml of 0.6 N methanolic HCl in a teflon-lined screw-capped tube, and heated at 75°C in a block heater for 1 hr. After allowing the tubes to cool, 0.5 ml of water was added and the DMAs were extracted with three 5 ml portions of petroleum ether. The DMAs in petroleum ether were concentrated under N_2 and stored in a small volume of petroleum ether at –20°C until use.

2.13 Data Analysis

Data were expressed as mean ± standard error, wherever possible. Significant differences between WS and WCS, and WCS and PGCS were performed using the GraphPad Instat software. The following tests were used: Analysis of Variance (ANOVA), the Tukey-Kramer Multiple Comparison test, and the t-test.

CHAPTER THREE

RESULTS

3.1 Preparation of Washed Non- and Capacitated, and PGC Capacitated Epididymal Mouse Sperm

The number of epididymal sperm retrieved from one mouse was 35.4 ± 8.6 million sperm (n = 38). Approximately 30 to 40% of these sperm showed weak motility, which increased slightly to about 50%, with more vigorous beats and higher speed of movement, upon suspension in BSA-containing capacitating medium. The sperm pellet obtained following centrifugation through Percoll contained 8.3 ± 1.2 million sperm per mouse (n = 72) and represented about 20% of the total sperm retrievable per mouse. About 85 to 90% of PGCS sperm exhibited hyper-activated motility patterns.

Variations in sperm number of up to 40% were obtained when the same sample was counted more than once within 5 to 10 min (for example, 31 ± 11 million sperm). This large variation may have been the sum of many factors including the inherent imprecision of manual counting, large scale dilution, and the fact that sperm tend to stick to the surface of tubes if they are left to sit for too long. Sperm prepared in the absence of BSA also seemed to have a greater variation in counting than sperm prepared with BSA. To avoid this problem, sperm were counted immediately following suspension in medium.

3.1.1 Sperm-Egg Binding of Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

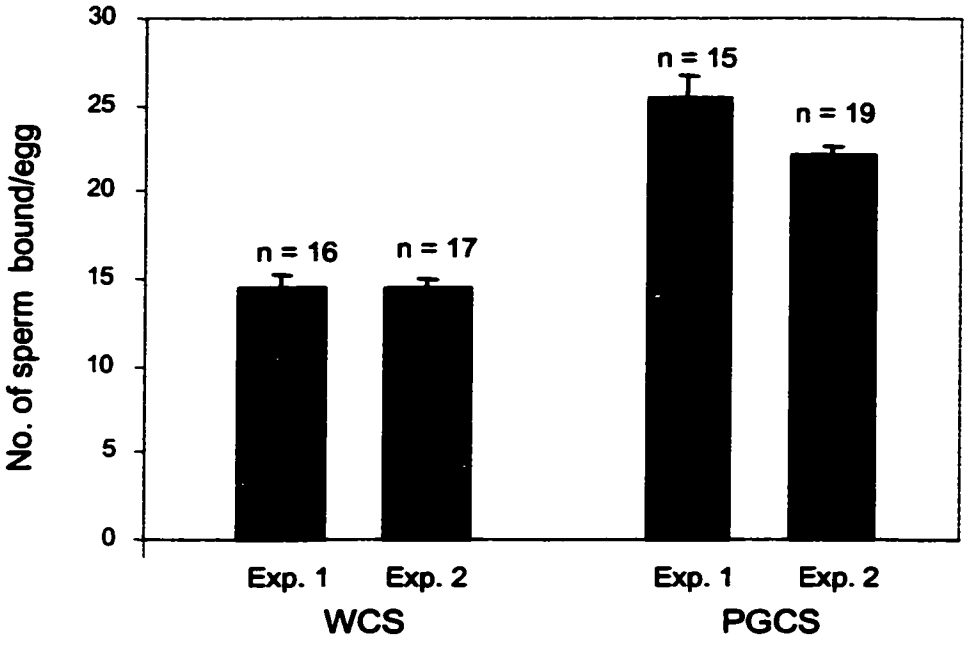
WCS and PGCS showed a significant difference in their abilities to bind to the egg ZP. The number of WCS bound per egg was 14.5 ± 2.6 (Experiment 1, n = 17) and 14.4 ± 2.6 (Experiment 2, n = 18), while the number of PGCS bound per egg was 25.4 ± 5.4 (Experiment 1, n = 16) and 22.2 ± 2.1 (Experiment 2, n = 20) ($p < 0.0001$) (Figure 3.1A). The pattern of binding differed between the two preparations with WCS binding in clumps, while PGCS bound uniformly around the egg in a sun-ray-like pattern (Figure 3.1B).

3.2 Analysis of DNA Levels and the Major Lipid Classes in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

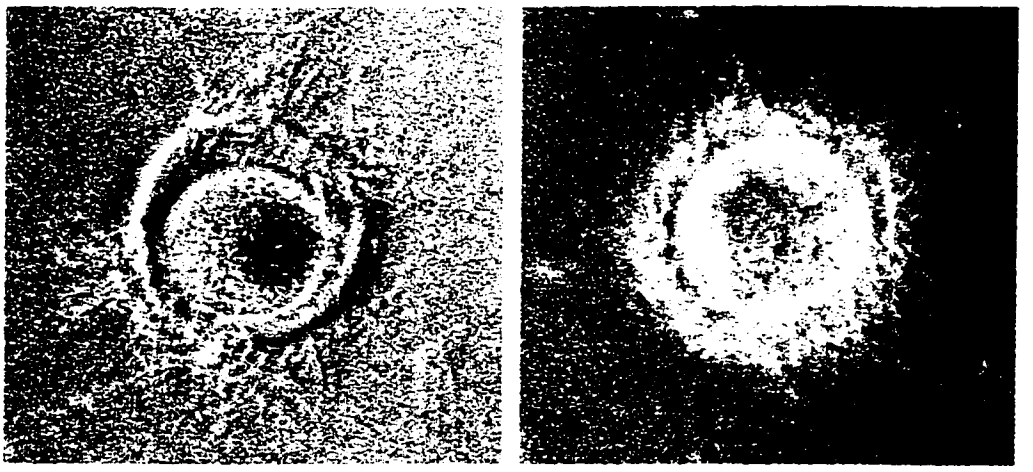
Statistical comparisons were made between washed sperm before and after capacitation. Sperm prepared by PGC consisted of the population that was mainly motile and having normal morphology (Tanphaichitr et al., 1988). WS contained all populations having both normal and abnormal morphology, therefore, WS were not compared with PGCS, since there may be differences in their physiology and biochemistry. However, WCS and PGCS were compared to determine whether any differences in capacitation methods existed.

Figure 3.1: A) Binding of WCS and PGCS to the ZP of mouse eggs. Results are expressed as the average ± standard error of the number of sperm bound/ZP in each experiment. PGCS bound significantly more sperm per egg than WCS ($p < 0.0001$). B) Binding patterns of sperm to the egg. (i) WCS bound in clumps around the egg, while (ii) PGCS bound uniformly around the egg in a sun-ray-like pattern.

A)


30
25
20
15
10
5
0
No. of sperm bound/egg
n = 16
n = 17
n = 15
n = 19
Exp. 1
Exp. 2
Exp. 1
Exp. 2
WCS
PGCS

B)


WCS
PGCS

3.2.1 DNA Levels

DNA levels were quantified to determine whether the amount of DNA per sperm cell was consistent. The meiotic division of spermatogenic cells during spermatogenesis results in each cell containing one set of DNA chromosomes that is unaltered during storage of mature sperm in the caudae epididymes; however, meiosis can also be abnormal and result in aneuploidy in some of the cells (Abruzzo and Hassold, 1995; Wyrobek et al., 1995). In the case of WS, which contain all populations of cells, it might have been expected that some sperm contained aneuploidic DNA, and thus, would affect the amount of DNA. Since PGC selects for the sperm population that is mature and highly motile, it was expected that aneuploidy would not occur.

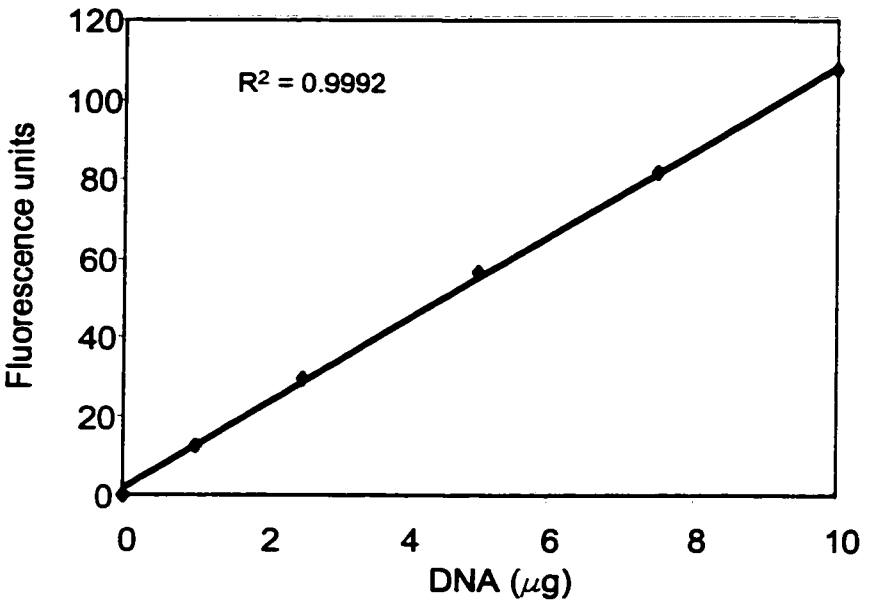
Sperm DNA was quantified against a standard curve of calf thymus DNA (Figure 3.2A). The level of DNA per sperm cell remained consistent regardless of the cell preparation. The amount of DNA in WS, WCS and PGCS was 4.2 ± 0.4, 4.0 ± 0.4 and 4.1 ± 0.2 pg DNA/sperm, respectively (Figure 3.2B). Scattering of DNA levels was within the same range for the two washed preparations, while the levels of DNA in PGCS scattered within a smaller range. Since the level of DNA per sperm was consistent in all three sample preparations, it was used to accurately express the lipid data instead of cell number.

3.2.2 Levels of Cholesterol and Phospholipid in Sperm

Cholesterol levels were analyzed by a spectrofluorometric assay and quantified against a standard curve of cholesterol (Figure 3.3). The level of cholesterol in extracted sperm lipids was lower in WCS compared to WS (i.e., 0.338 ± 0.068 vs. 0.430 ± 0.075 nmole/μg DNA, respectively); however, this decrease was not significant (Figure 3.4).

Figure 3.2: A) DNA assay standard curve. Calf thymus DNA was used to construct a standard curve and the range of fluorescence units was very consistent from assay to assay. A linear relationship was observed up to 10 μg of DNA, and the sensitivity of the assay was 1 μg DNA. Sperm aliquots were assayed in the range of about 4 to 5 μg DNA. B) Levels of DNA in WS, WCS and PGCS.

A)


120
100
80
60
40
20
0
Fluorescence units
R² = 0.9992
0
2
4
6
8
10
DNA (μg)

B)

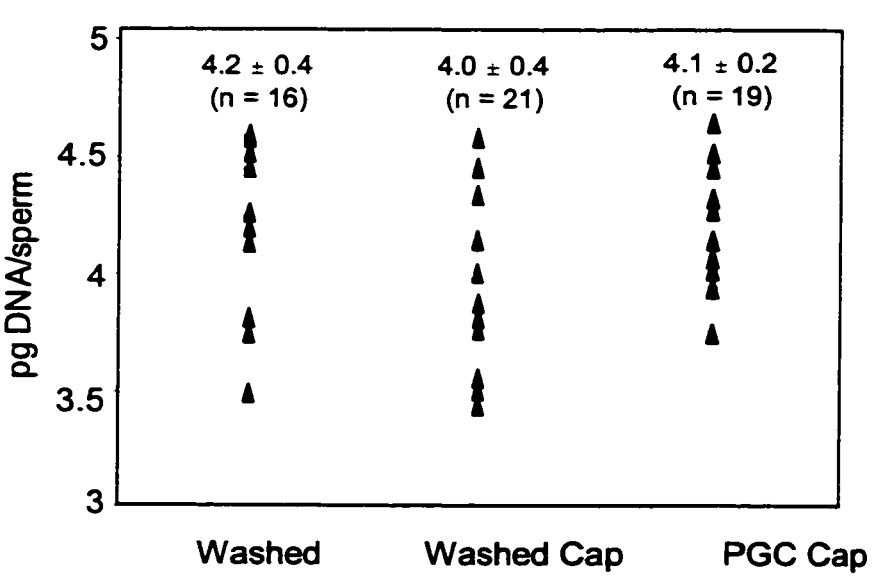

5
4.5
4
3.5
3
pg DNA/sperm
4.2 ± 0.4
(n = 16)
4.0 ± 0.4
(n = 21)
4.1 ± 0.2
(n = 19)
Washed
Washed Cap
PGC Cap

Figure 3.3: Cholesterol assay standard curve. Cholesterol was used to construct a standard curve and the range of fluorescence units was consistent from assay to assay. The relationship between the amount of cholesterol and the fluorescence units was consistently linear in the range up to 0.5 μg cholesterol. The sensitivity of the assay was 0.05 μg. Sperm lipids were measured in the range from 0.2 to 0.4 μg.

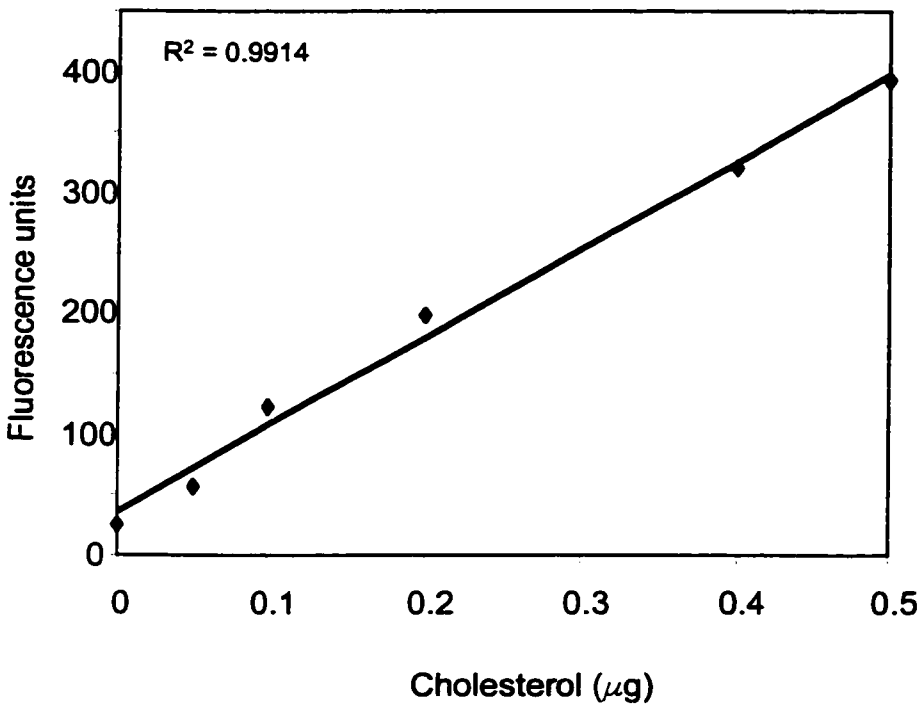
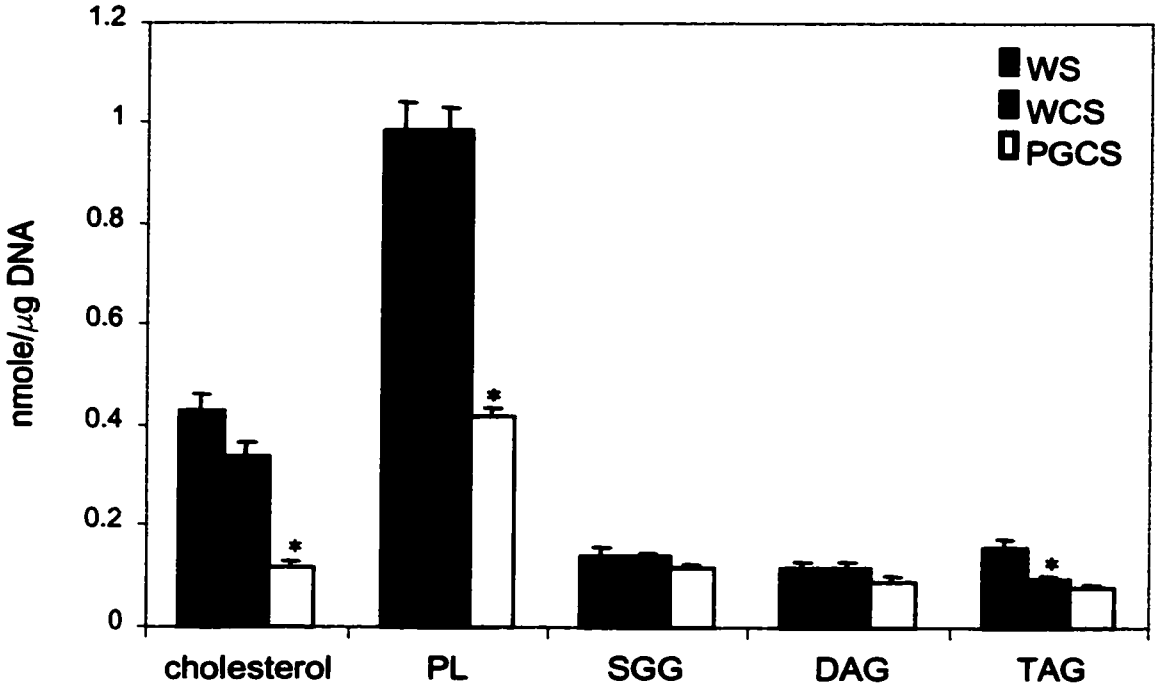

R² = 0.9914
400
300
200
100
0
Fluorescence units
0
0.1
0.2
0.3
0.4
0.5
Cholesterol (μg)

Figure 3.4: Levels of the major lipid classes in washed, washed capacitated and PGC capacitated epididymal mouse sperm. The levels of cholesterol, PL, DAG and TAG were analysed 5 times, while SGG was analysed 3 times. The results are expressed as an mean ± standard deviation. The level of TAG in WCS was significantly lower than in WS ($p < 0.05$), and the levels of cholesterol ($p < 0.001$) and PL ($p < 0.001$) in PGCS were significantly lower than in WS (*).


1.2
1
0.8
0.6
0.4
0.2
0
nmole/μg DNA
WS
WCS
PGCS
*
*
*
cholesterol
PL
SGG
DAG
TAG

PGCS had a significantly lower level of cholesterol compared to WCS (0.120 ± 0.017 vs. 0.338 ± 0.068 nmole/μg DNA, respectively; $p < 0.001$).

Phospholipid (PL) levels were analysed using a colorimetric assay and quantified against a standard curve that was constructed using KH_2PO_4 (Figure 3.5). A standard curve using PC was similar to that of KH_2PO_4. Lipids were quantified as μg P, but since there is one P atom in each PL molecule, 1 nmol P is equal to 1 nmol PL. Cardiolipin is the only PL that contains two P's, however, it is not present at a great amount in sperm (Hall et al., 1991; Rana et al., 1991; Nikolopoulou et al., 1985; Parks and Hammerstedt, 1985; Evans and Setchell, 1979; Poulos and White, 1973) and was not detected by HPTLC (Figure 3.10).

The amount of PL remained unchanged in WS and WCS and was present as 0.986 ± 0.125 and 0.987 ± 0.101 nmole/μg DNA, respectively (Figure 3.4). In PGCS, the amount of PL was almost 60% lower than in the WS (0.419 ± 0.035 nmole/μg DNA). The level of PL in PGCS was significantly lower than in WCS ($p < 0.001$).

The cholesterol:PL molar ratio was lower in WCS compared to WS and decreased from 0.44 ± 0.04 to 0.34 ± 0.04, but was not significantly different. In PGCS, the cholesterol:PL molar ratio was further decreased to 0.29 ± 0.02, but was not significantly lower than in WCS.

3.2.3 Levels of SGG

Analysis of SGG was based on the formation of a blue-colored complex between the cationic dye Azure A to sulfolipids. A standard curve of SGC (Sigma) was used to determine the amount of SGG (Figure 3.6). SGG and SGC are structural analogues. Both sulfoglycolipids contain two hydrophobic chains and have a single galactosylsulfate

Figure 3.5: Phospholipid assay standard curve constructed using KH_2PO_4. The relationship between the amount of PL and the absorbance (650 nm) was linear in the range up to 0.1 μg PL, and the sensitivity of the assay was 0.015 μg. Sperm lipid samples were measured in the range from 0.04 to 0.08 μg.

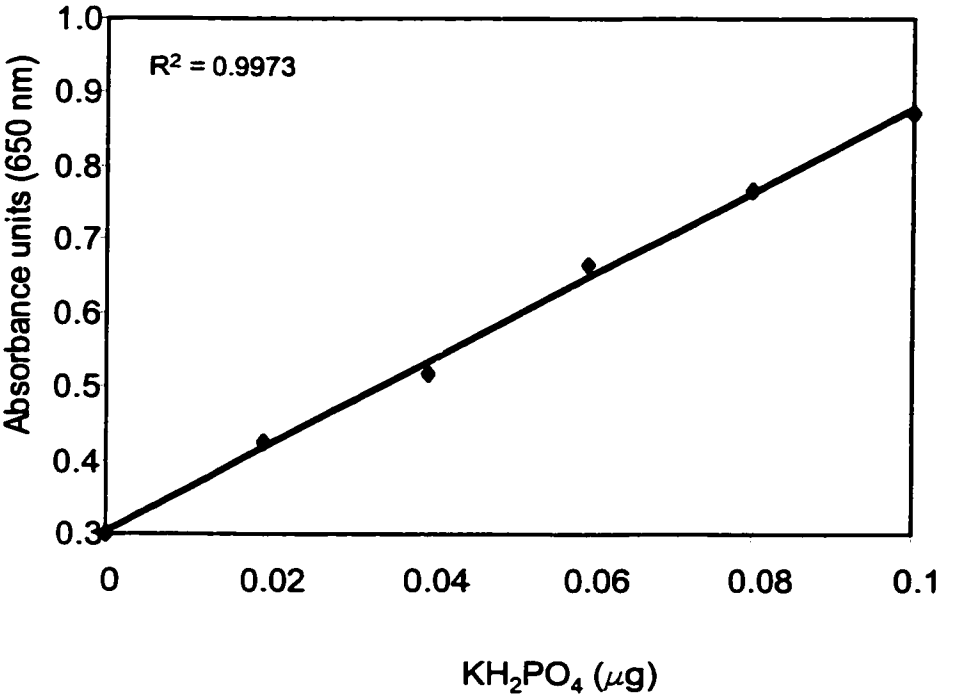
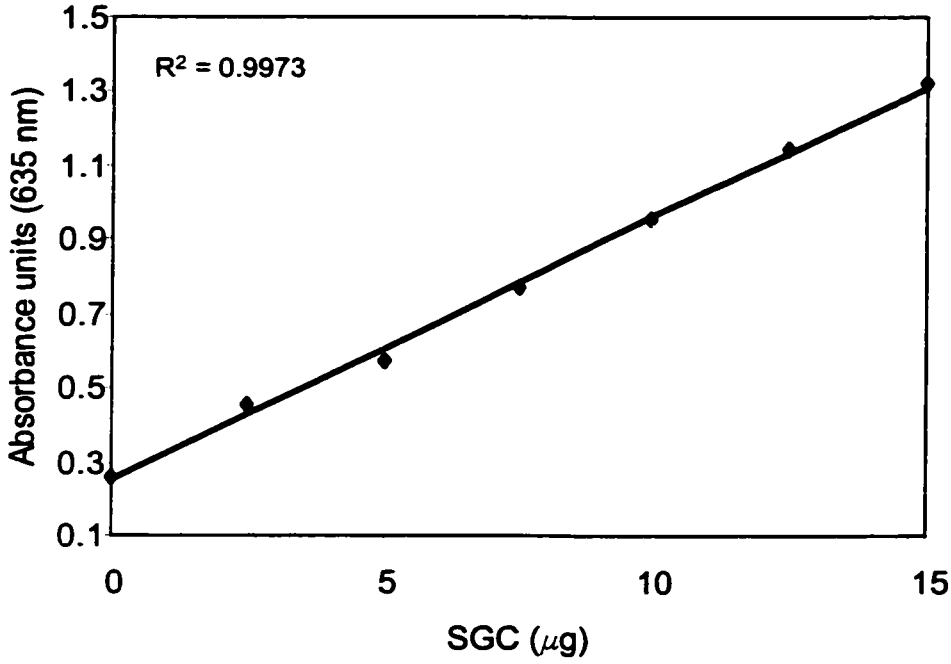

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
R² = 0.9973
Absorbance units (650 nm)
0
0.02
0.04
0.06
0.08
0.1
KH2PO4 (μg)

Figure 3.6: Sulfolipid assay standard curve constructed using SGC. The standard curve was linear in the range from 0 to 15 μg of SGC, and the sensitivity of the assay was 1.5 μg. Sperm lipids were measured in the range from 5 to 10 μg.


1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
R² = 0.9973
Absorbance units (635 nm)
0
5
10
15
SGC (μg)

moiety as a polar head group. Since SGC is commercially available, it was used as a standard in the SGG quantification assay. There was no significant difference in the amount of SGG in WS and WCS (0.139 ± 0.027 and 0.143 ± 0.006 nmole/μg DNA, respectively) (Figure 3.4). In the PGCS, SGG was present at a level of 0.120 ± 0.006 nmole/μg DNA.

3.2.4 Levels of DAG and TAG

DAG and TAG were separated by HPTLC using a solvent system of petroleum ether:ethyl ether:acetic acid (80:20:1, v/v/v), which effectively separated DAG and TAG, giving R_f values of 0.22 and 0.71 to 0.73, respectively (Figure 3.7). TAG frequently migrated on an angle such that each TAG spot had a slightly different R_f value. This was observed with each analysis, and may have resulted from the solvent running unevenly at the top of the plate or evaporation of solvent closer to the edges of the plate resulting in alteration of solvent composition. Cholesterol migrated close to DAG, with an R_f value of 0.25, and was applied to each plate to clearly differentiate it from DAG. Sperm DAG and TAG were quantified by densitometric analysis and their amounts were determined from standard curves of DAG and TAG (0.25 to 2.5 μg) (Figure 3.8) that were chromatographed on the same HPTLC plate. It was interesting to note that, although cholesterol was not analyzed densitometrically, it was possible to see a decrease in the intensity of the cholesterol spot in PGC cap sperm compared to the washed samples, which agreed with the results obtained using the spectrofluorometric assay (Figure 3.4). The lipids remaining at the origin represented polar lipids, such as PLs and glycolipids, which did not migrate in this solvent system.

Figure 3.7: HPTLC separation and densitometric analysis of DAG and TAG in washed, washed capacitated and PGC capacitated epididymal mouse sperm lipids. Neutral lipids were separated using a solvent system of petroleum ether:ethyl ether: acetic acid (80:20:1, v/v/v) and visualized using Coomassie blue staining. DAG and TAG were quantified by densitometric analysis and the amounts in the samples were determined by comparison with standard curves chromatographed on the same plate. The DAG standard was a mixture of the 1,2- and 1,3-isomers, which were both visible following staining and are labelled in the figure.

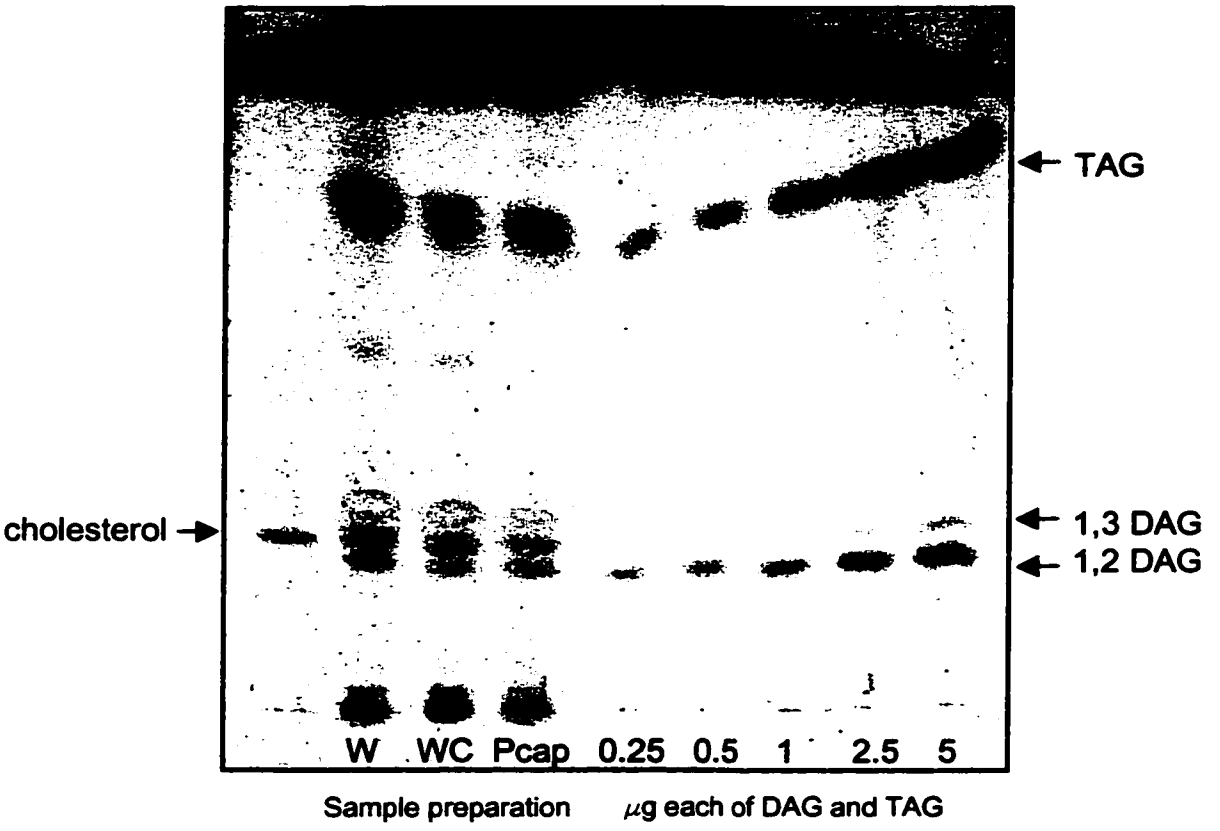
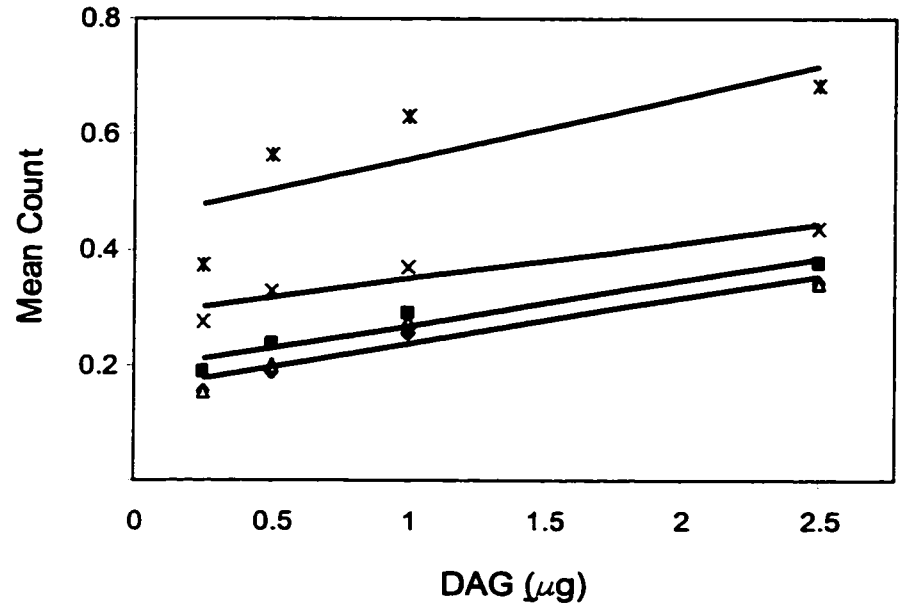
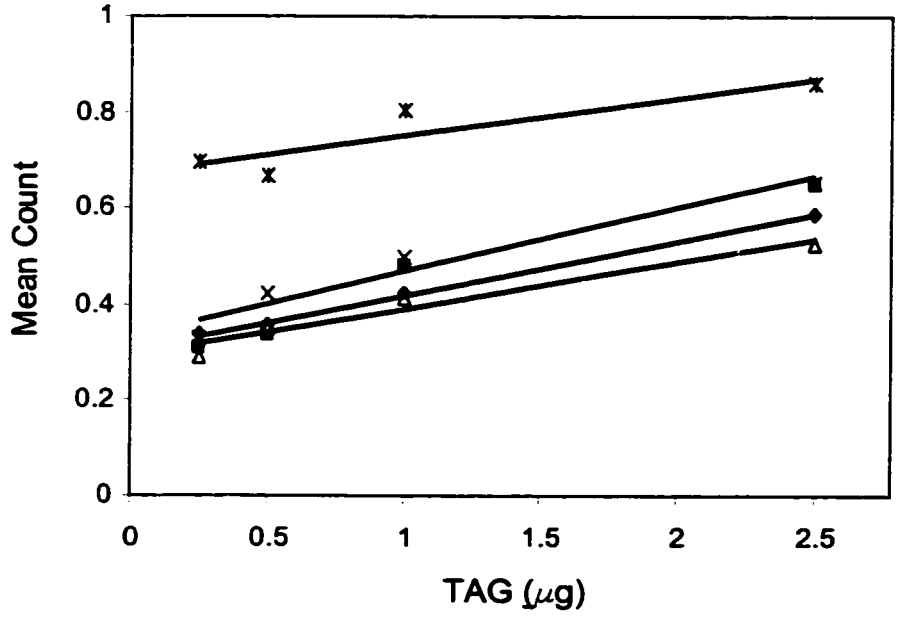

TAG
cholesterol
1,3 DAG
1,2 DAG
W
WC
Pcap
0.25
0.5
1
2.5
5
Sample preparation
μg each of DAG and TAG

Figure 3.8: A) DAG standard curve. DAG was quantified by HPTLC, Coomassie blue staining and densitometric analysis (Nakamura and Handa, 1984). Quantification was based on comparison of the samples with standard curve co-chromatographed on the same HPTLC plate. Samples were consistently measured between 1 to 2 μg on the standard curve. B) TAG standard curve. TAG was quantified by HPTLC as described for DAG. The standard curve used for quantification was a combination of five analyses. Samples were consistently measured between 1 to 2 μg on the standard curve. Both standard curves are an average of 5 assays.

A)


0.8
0.6
0.4
0.2
Mean Count
0
0.5
1
1.5
2
2.5
DAG (μg)

B)


1
0.8
0.6
0.4
0.2
0
Mean Count
0
0.5
1
1.5
2
2.5
TAG (μg)

linear in this range. The levels of DAG in all three samples were 0.119 ± 0.028, 0.116 ± 0.034, and 0.091 ± 0.020 nmole/μg in WS, WCS and PGCS, respectively (Figure 3.4). The levels of TAG in the three samples were 0.160 ± 0.037, 0.096 ± 0.014, and 0.079 ± 0.012 nmole/μg. This decrease of TAG levels following capacitation in the washed samples was visibly seen on the HPTLC plate (Figure 3.7) and was significantly different ($p < 0.05$).

3.2.5 Molar Distribution of Cholesterol, PL, SGG, DAG and TAG

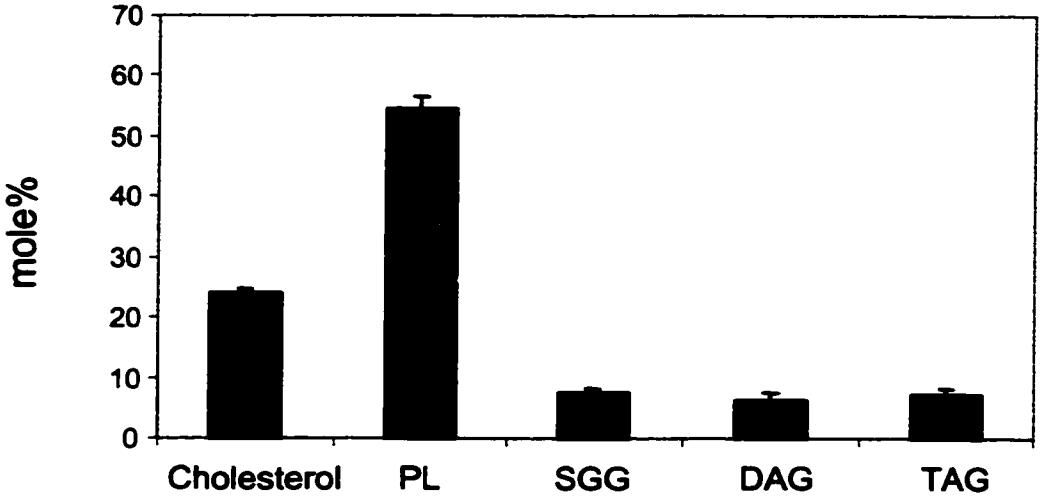
There were no significant differences in the molar distributions of cholesterol or PL in WS, WCS and PGCS (Figure 3.9). In both WS and WCS, the distribution of SGG was about 8 mole% of the total lipids (Figure 3.9A and B), while SGG constituted almost 14 mole% of the total lipids in PGCS (Figure 3.9C).

3.3 Distribution and levels of the Major PL Classes

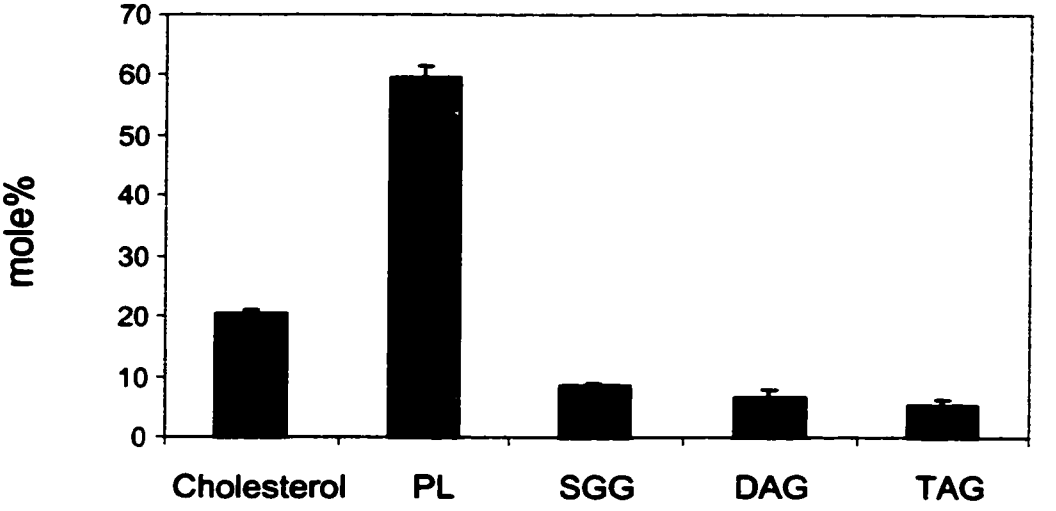
In all three sperm lipid samples, the only PL classes that were detected by either orcinol/acid charring or Coomassie blue staining were PC, PE and SPH, having R_f's of 0.18, 0.68 and 0.11, respectively. Lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), PS, PI and cardiolipin were not detected. The detected PL classes were assumed as 100%. The distribution of PC, PE and SPH appeared to be similar in the three sperm samples, i.e., in WS, 60.0, 34.6 and 5.37%, respectively, in WCS, 55.3, 39.9 and 4.81%, respectively, and in PGCS, 58.8, 36.4 and 4.83%, respectively. The levels of PC, PE and SPH are shown in Table 3.1. The presence of other lipid spots, which stained positive (blue) with orcinol (Figure 3.10A), were detected and presumed to be glycolipids.

Figure 3.9: Molar distribution of the major lipid classes in WS (A), WCS (B) and PGCS (C). The distributions of cholesterol, PL, DAG and TAG were determined from 5 samples, while SGG was determined from 3 samples. The results are expressed as mean ± standard deviation.

A)


70
60
50
40
30
20
10
0
mole%
Cholesterol
PL
SGG
DAG
TAG

B)


70
60
50
40
30
20
10
0
mole%
Cholesterol
PL
SGG
DAG
TAG

C)

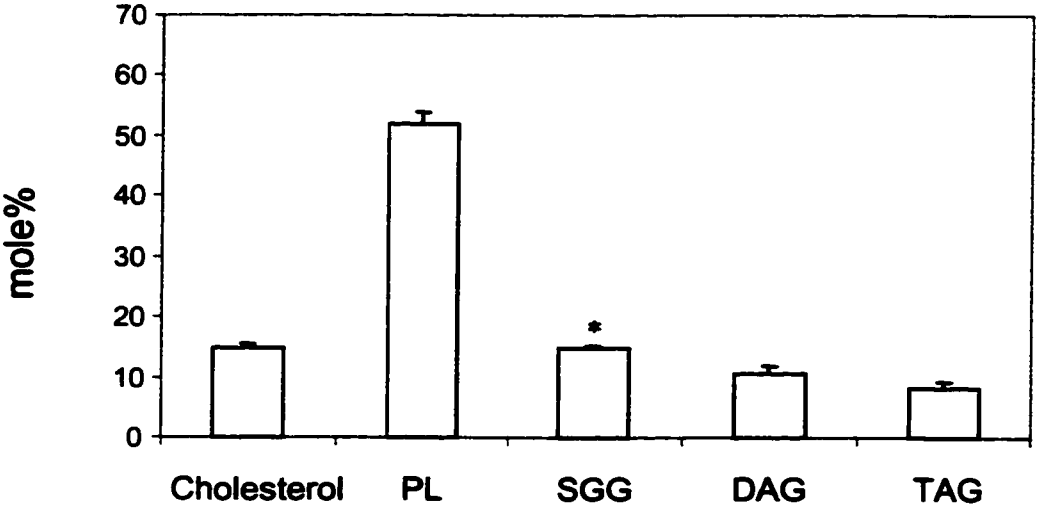
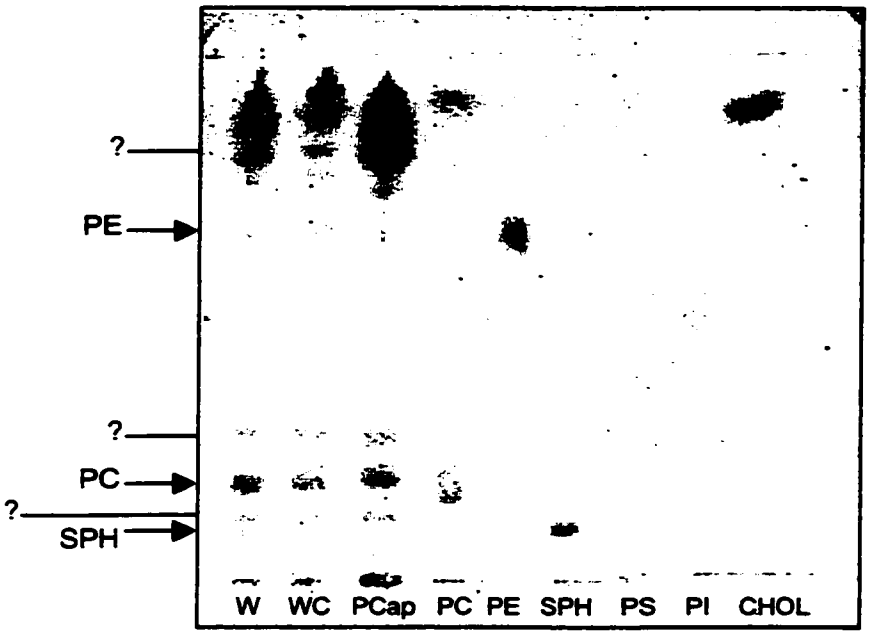

70
60
50
40
30
20
10
0
mole%
*
Cholesterol
PL
SGG
DAG
TAG

Figure 3.10: HPTLC separation and densitometric analysis of the major PL classes in washed, washed capacitated and PGC capacitated epididymal mouse sperm lipid. Lipids were separated using a solvent system of chloroform:methanol:acetic acid:water (50:37.5:3.5:2, v/v/v/v) and first stained with orcinol and acid charring (A). The plate was further stained with Coomassie blue (B), and the distribution of PC, PE and SPH was determined by densitometric analysis using Molecular Analyst software. Unknown spots that stained blue with orcinol, and were presumed to be glycolipids, are labelled with a '?'.

A



B

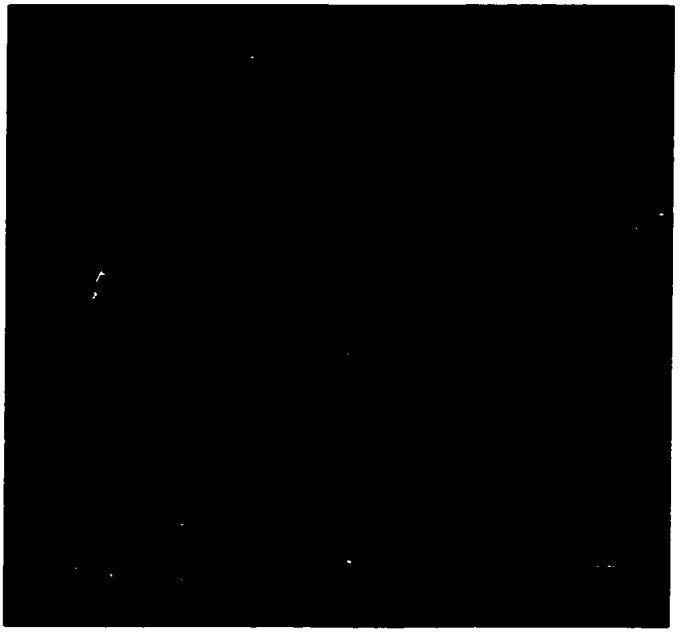


Table 3.1: Levels[1] of PC, PE and SPH in WS, WCS and PGCS

	PC[2]	PE[2]	SPH[2]
WC	0.592	0.341	0.053
WCS	0.546	0.394	0.046
PGCS	0.246	0.152	0.020

[1]Levels were calculated based on the % distribution of PC, PE and SPH determined by densitometric analysis of the lipid spots on the HPTLC plate, and the levels of PL in each sample as determined by the PL assay (Table 3.4)
[2]Levels of PC, PE and SPH expressed as nmole/μg DNA.

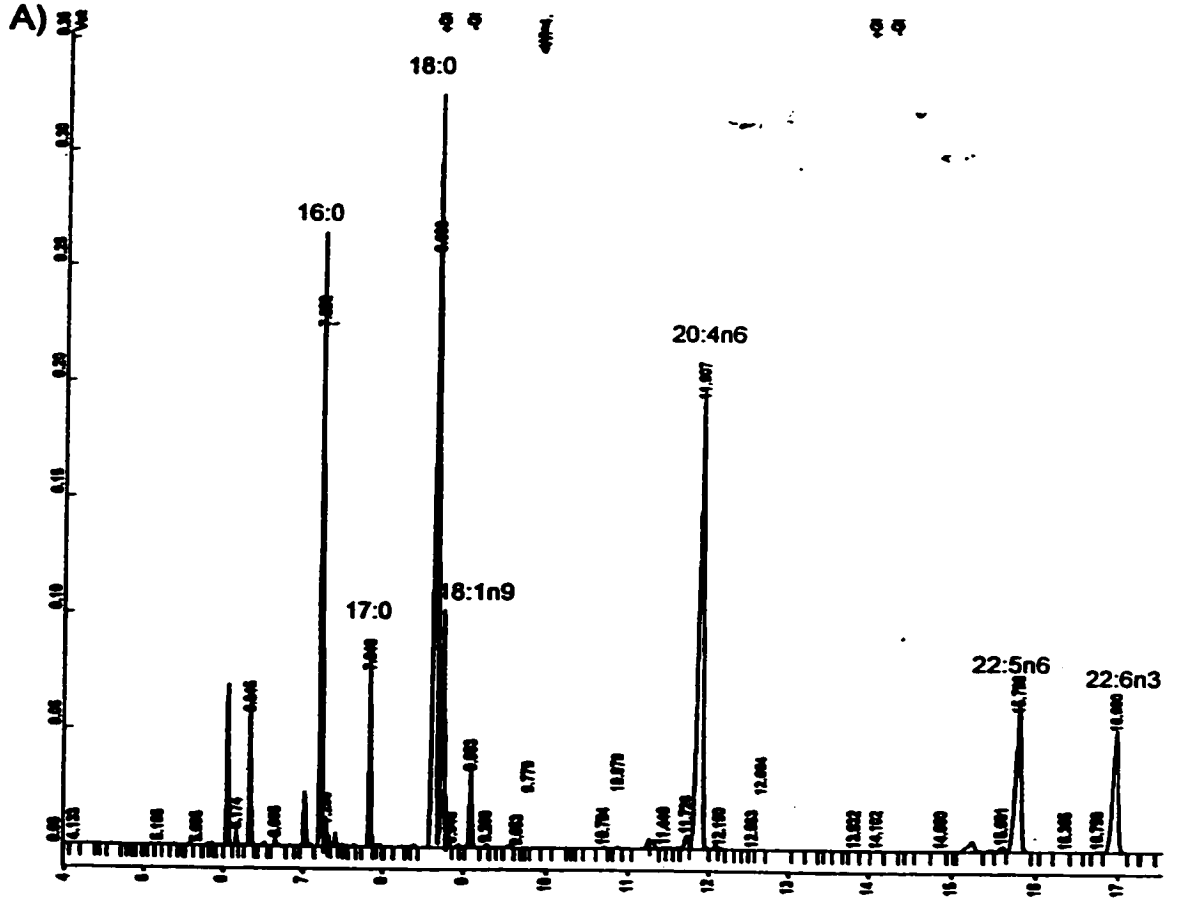
3.4 Levels of the Major Fatty Acyl Chains of PC and PE in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

Previous work performed in this laboratory determined that there was no difference in the cholesterol:PL molar ratio following incubation of either washed or PGC sperm in albumin-containing capacitating medium (Tanphaichitr et al., 1996). This led to the hypothesis that there might be a modification of the fatty acyl chain composition of PC and PE, the major lipid classes in the sperm membrane, which might influence membrane fluidity during these two in vitro capacitation procedures.

Sample GC chromatograms for PC- and PE-bound FAMEs of WC, WCS, and PGCS (as performed by the Lipid Analytical Laboratories in collaboration with Dr. Bruce Holub at the University of Guelph) are shown in Figures 3.11 to 3.13. Sperm lipids have been shown to contain a high proportion of plasmalogens (Poulos et al., 1973; Alvarez et al., 1985; Nikolopoulou et al., 1985; Parks and Lynch, 1992; Lin et al., 1993; Brouwers et al., 1998), and thus contain hydrocarbon chains linked via an alk-1-enyl ether linkage, which do not form FAMEs. Instead, these fatty alcohols generate dimethyl acetals (DMAs) when subjected to acid methanolysis (Kates, 1986) and were not analyzed by GC at the University of Guelph. New samples were prepared and sent to Health Canada for FAME and DMA analysis by GC-MS, which was performed in collaboration with Dr. Renaud Vincent by Dr. Premkumari Kumarathasan.

Quantification of FAMEs was performed by comparison of the area under each peak with that of a known amount of 17:0 that was added to each sample and the results

Figure 3.11: GC chromatogram of the major PC- (A) and PE- (B) bound FAMEs in WC. The x-axis represents the time for sample running (in minutes), and the y-axis represents volts (A) and millivolts (B). Each major peak is labeled for its corresponding FAME as determined by comparison with the retention times of authentic FAME standards.


A)
18:0
16:0
20:4n6
17:0
18:1n9
22:5n6
22:6n3

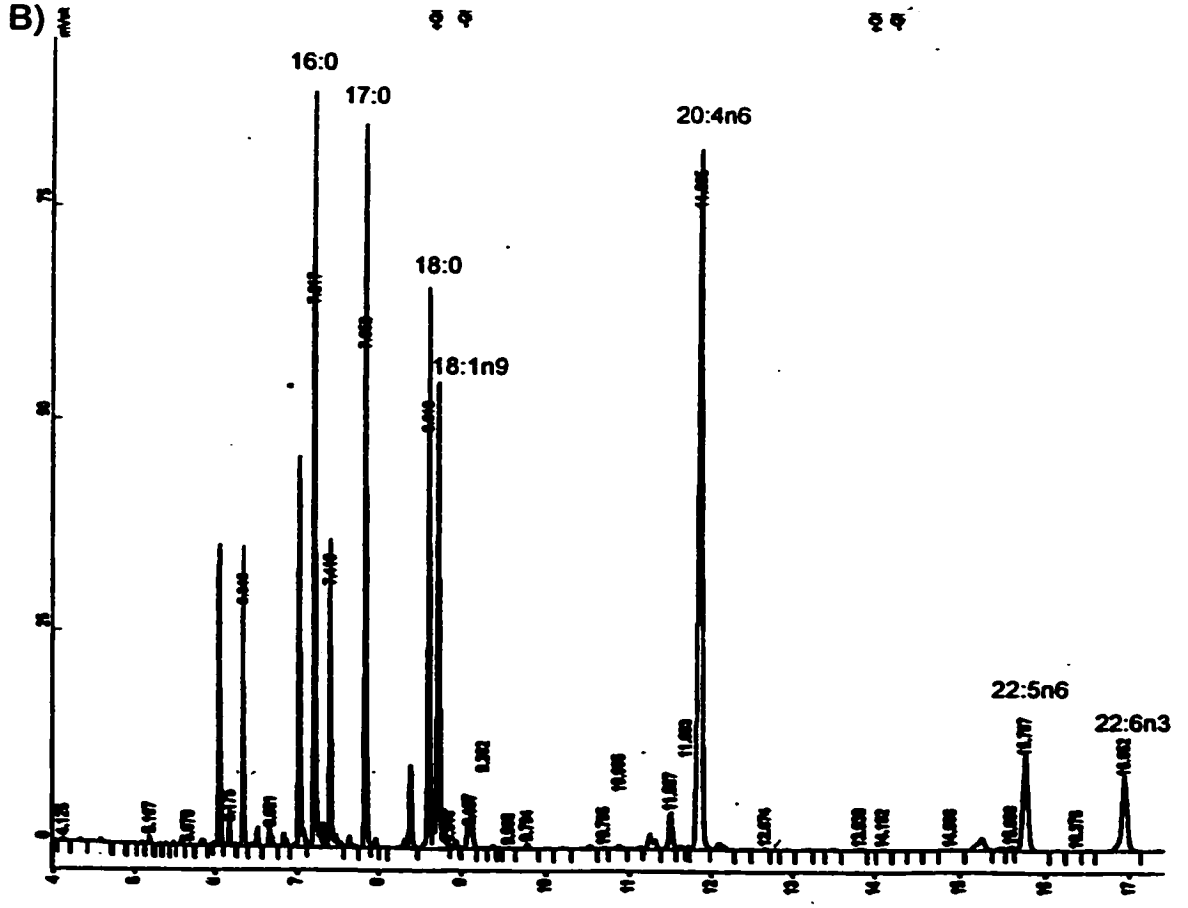
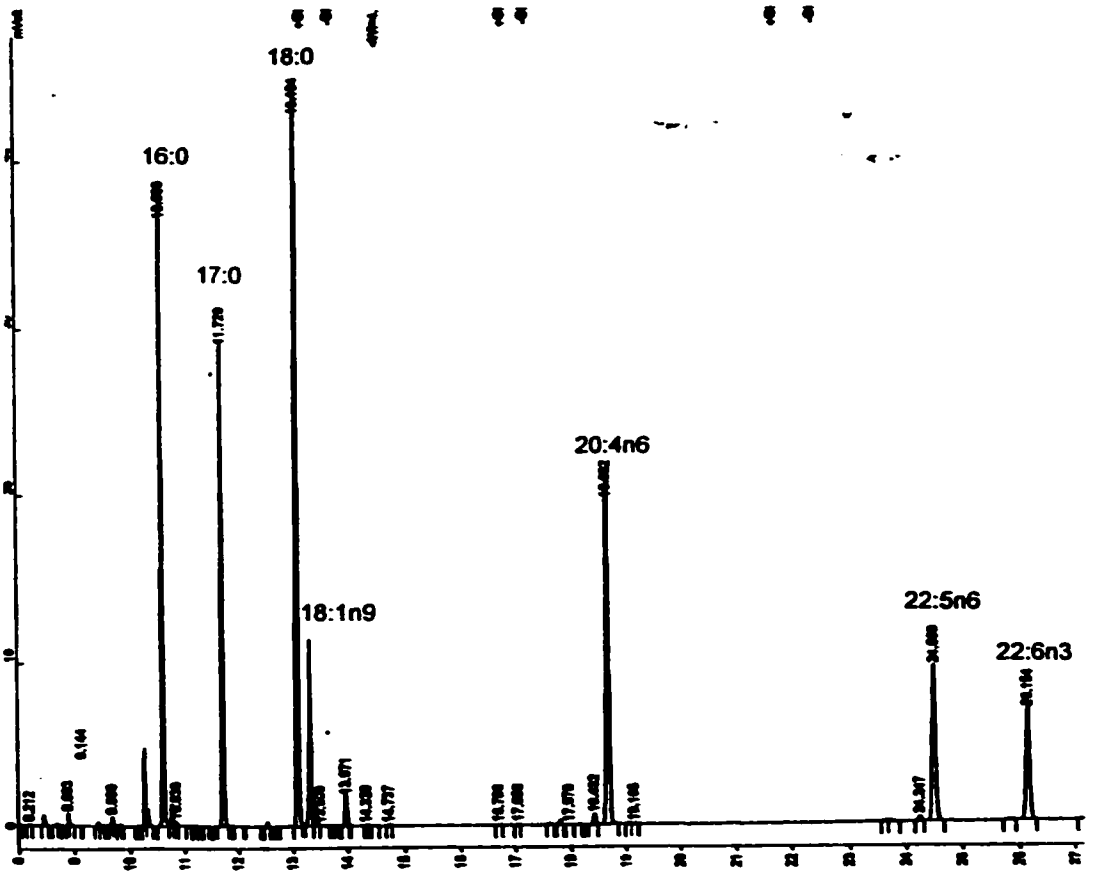

B)
16:0
17:0
20:4n6
18:0
18:1n9
22:5n6
22:6n3

Figure 3.12: GC chromatogram of the major PC- (A) and PE- (B) bound FAMEs in WCS. The x-axis represents the time for sample running (in minutes), and the y-axis represents millivolts. Each major peak is labeled for its corresponding FAME as determined by comparison with the retention times of authentic FAME standards.

A)


18:0
16:0
17:0
20:4n6
18:1n9
22:5n6
22:6n3

B)

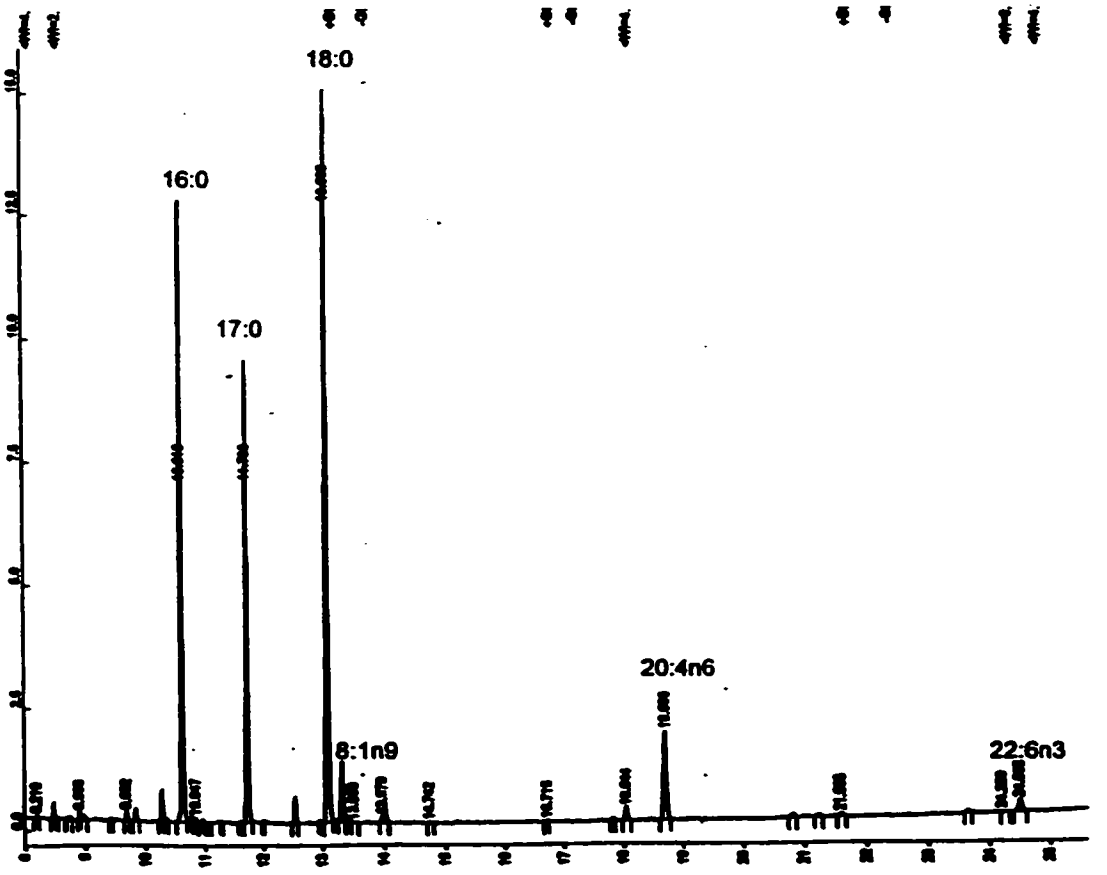
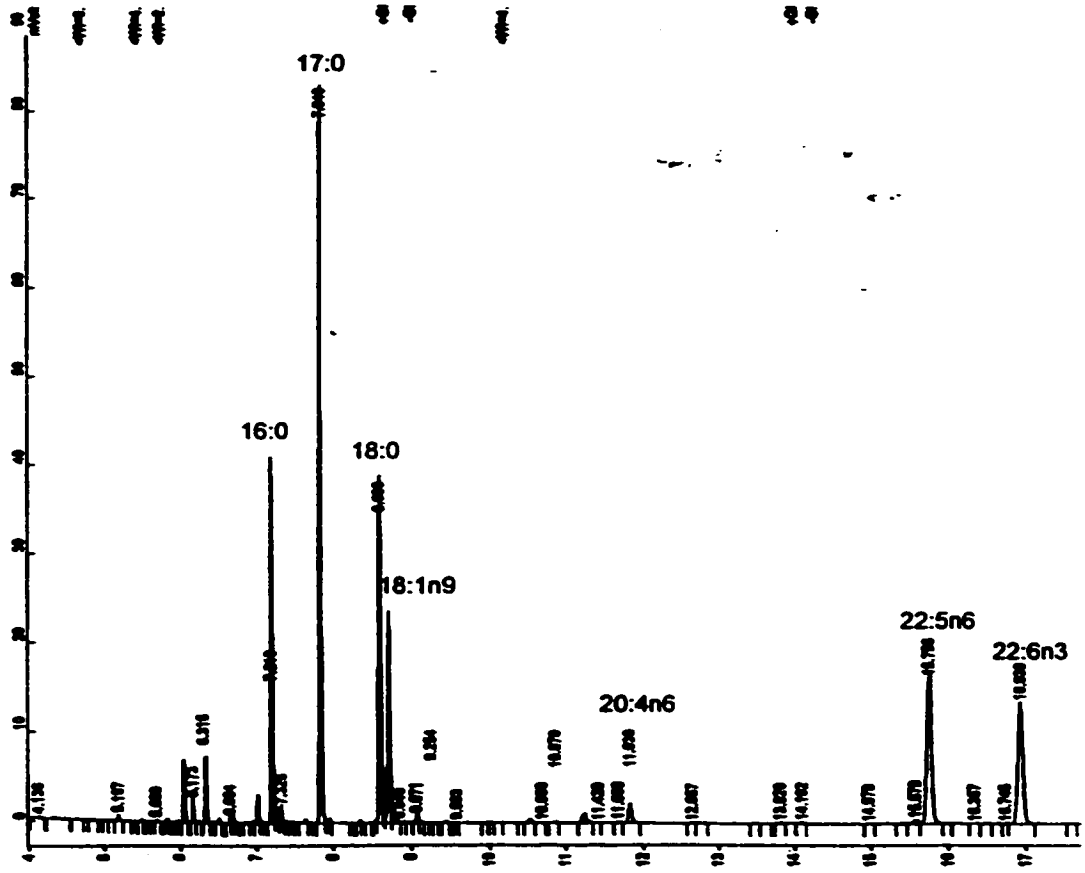
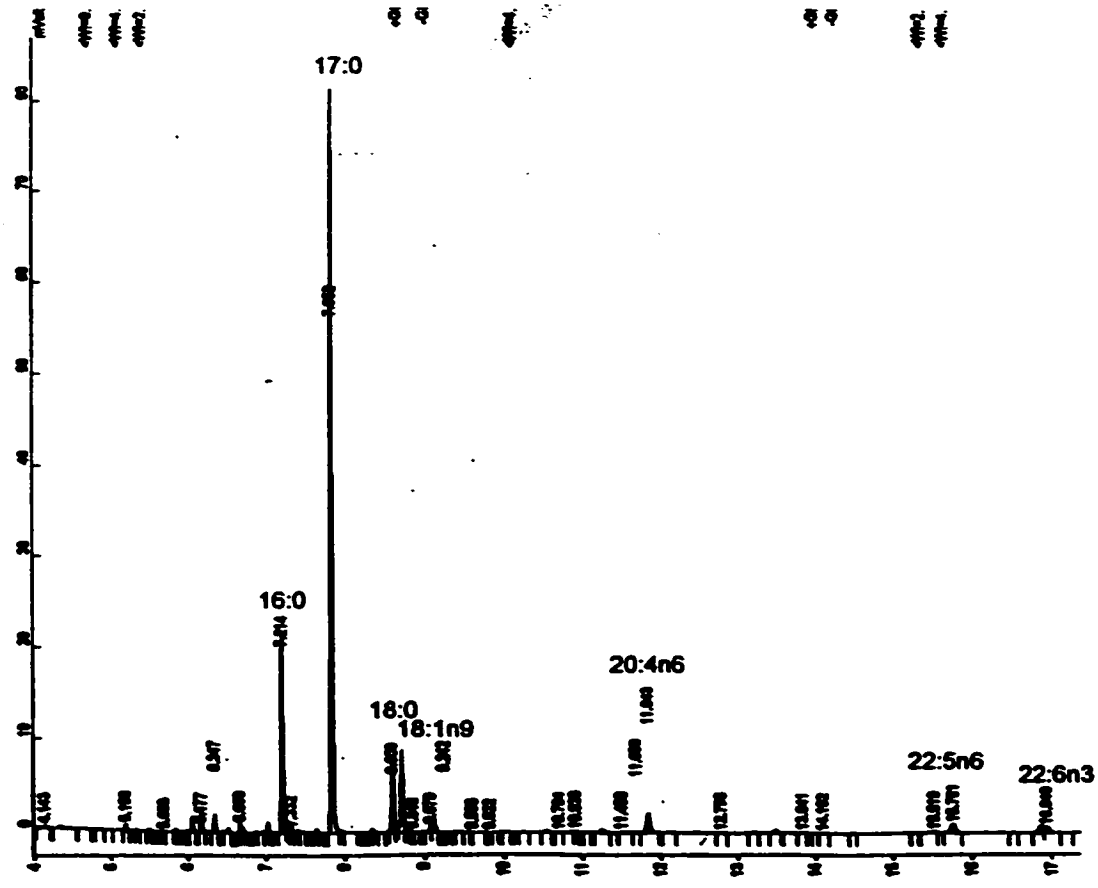

18:0
16:0
17:0
20:4n6
8:1n9
22:6n3

Figure 3.13: GC chromatogram of the major PC- (A) and PE- (B) bound FAMEs in PGCS. The x-axis represents the time for sample running (in minutes), and the y-axis represents millivolts. Each major peak is labeled for its corresponding FAME as determined by comparison with the retention times of authentic FAME standards.

A)


17:0
16:0
18:0
18:1n9
22:5n6
22:6n3
20:4n6

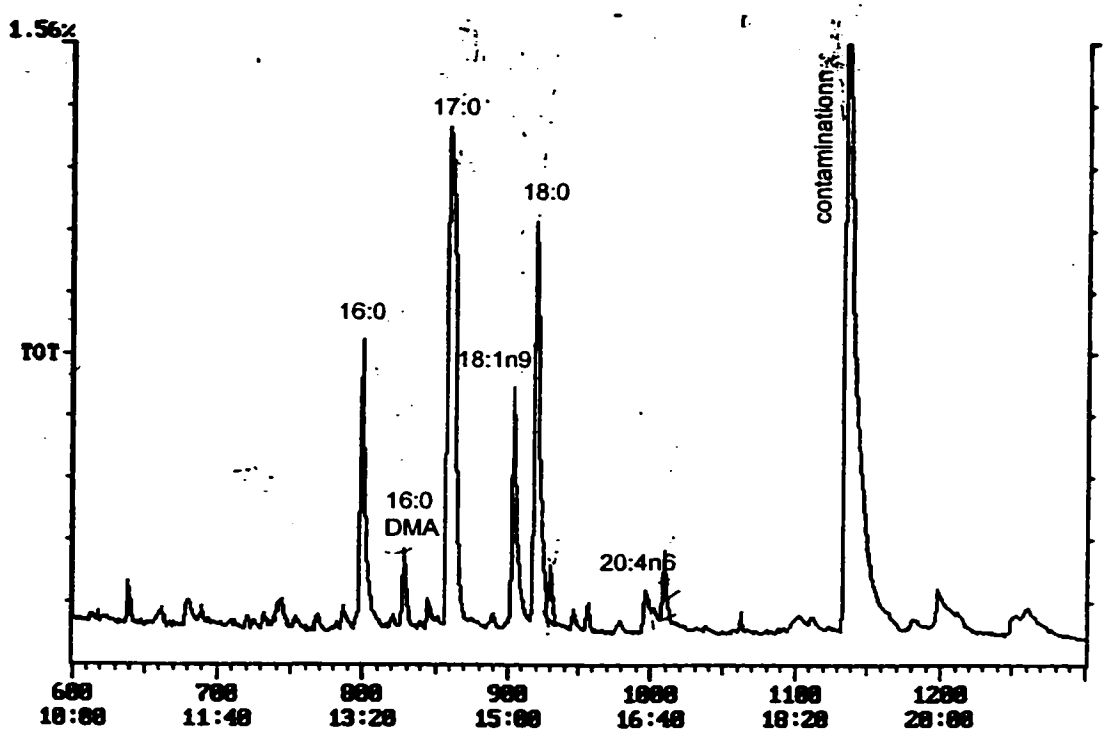
B)


17:0
16:0
20:4n6
18:0
18:1n9
22:5n6
22:6n3

were expressed as a molar distribution. Tables A.1 to A.9 in the Appendix show the raw data of the molar distribution for PC, PE and SPH of the three sperm preparations. The FAME analysis of WS was performed three times. The PC-bound fatty acyl chains had a maximum deviation of ± 10% in the major FAMEs, while the minor longer chain PUFAs had a maximum deviation of ± 25%. A maximum deviation of ± 30% was observed in the PE-bound fatty acyl chains. WCS lipids were analysed three times, but one analysis was very inconsistent with the other two and was discarded (i.e., these data were not included with the raw data). For the PC analyses of WCS, the two analyses had a maximum deviation of ± 10%. However, inconsistencies were obtained with PE, more specifically with the PUFAs, and may have occurred because the amount of lipid injected into the GC was too low to detect the FAMEs that were present at lesser amounts. A new sample of PE-bound FAMEs from WCS was prepared and sent to Health Canada for GC-MS analysis (Figure 3.14). PGCS were analysed twice and had a maximum deviation of ± 20%. PGCS were analysed twice because of the large number of sperm, and thus mice (about 80), that were required for one sample preparation. The amount of each FAME (nmole) per μg DNA was calculated based on the levels of PL (Figure 3.4) and the % distribution of PC, PE and SPH in each sample.

The FAME analyses of SPH for all samples showed large inconsistencies. In the solvent system used to separate the phospholipid classes, other lipids migrated very close to SPH, therefore, it is possible that the SPH scraped off the plate was not pure. Because of these inconsistencies, the SPH data were not plotted in graph form.

Figure 3.14: GC chromatogram of PE from WCS lipids as analyzed by GC-MS at Health Canada. The major FAME peaks are labeled and were determined by comparison with the retention times of authentic FAME standards and by MS.


1.56%
TOT
17:0
18:0
16:0
18:1n9
16:0
DMA
20:4n6
contamination
600
10:00
700
11:40
800
13:20
900
15:00
1000
16:40
1100
18:20
1200
20:00

The major SFAs in all three samples were 16:0 and 18:0. The major MUFA was 18:1n9, and the major PUFAs were 20:4n6, 22:5n6 and 22:6n3. The identity of 22:5n6, for which a standard was not available, was confirmed by MS-MS analysis in collaboration with Dr. Tom Brenna at Cornell University by Anthony Michaud (Figure 3.15). The *m/z* 286 and 270 ions are diagnostic for the n-6 isomer of 22:5.

3.4.1 Molar Distribution of the Major PC-Bound Fatty Acyl Chains in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

In PC of WCS, the SFAs 16:0 and 18:0 constituted about 15 and 30 mole%, respectively (Figure 3.16A). Almost 8.5 mole% was 18:1n9 and the major PUFA was 20:4n6, which made up about 27 mole%. 22:5n6 and 22:6n3 were present at about 8.5 and 8 mole%, respectively. In WCS, 16:0 and 18:0 were present at about 20 and 28 mole%, respectively (Figure 3.16B). 18:1n9 constituted about 8 mole% and the major PUFA was 20:4n6, which contributed about 21 mole%, while 22:5n6 and 22:6n3 were present at about 11 and 8 mole%, respectively.

In PGCS, 16:0 and 18:0 constituted about 21 and 23 mole%, respectively (Figure 3.16C). About 13 mole% was 18:1n9, and the major PUFAs were 22:5n6 and 22:6n3, which made up about 21 and 16. 5 mole%, respectively. 20:4n6 was the minor fatty acyl chain and was present as about 3.5 mole%.

In summary, there was an increased distribution in PC-bound 16:0 and a decreased distribution of 20:4n6 following capacitation in the washed samples. PGCS had a similar distribution of SFAs compared to WCS, but there was an increased distribution of 18:1n9, 22:5n6 and 22:6n3 in PGC cap sperm. The distribution of 20:4n6 in PGCS was much lower than in WCS.

Figure 3.15: MS-MS analysis of an unknown peak present in the GC chromatograms, which was confirmed to be 22:5n6. Analysis was performed in collaboration with Dr. Tom Brenna at Cornell University.

Scan 469 from c:\saturn\data\sperm\021000so.ms

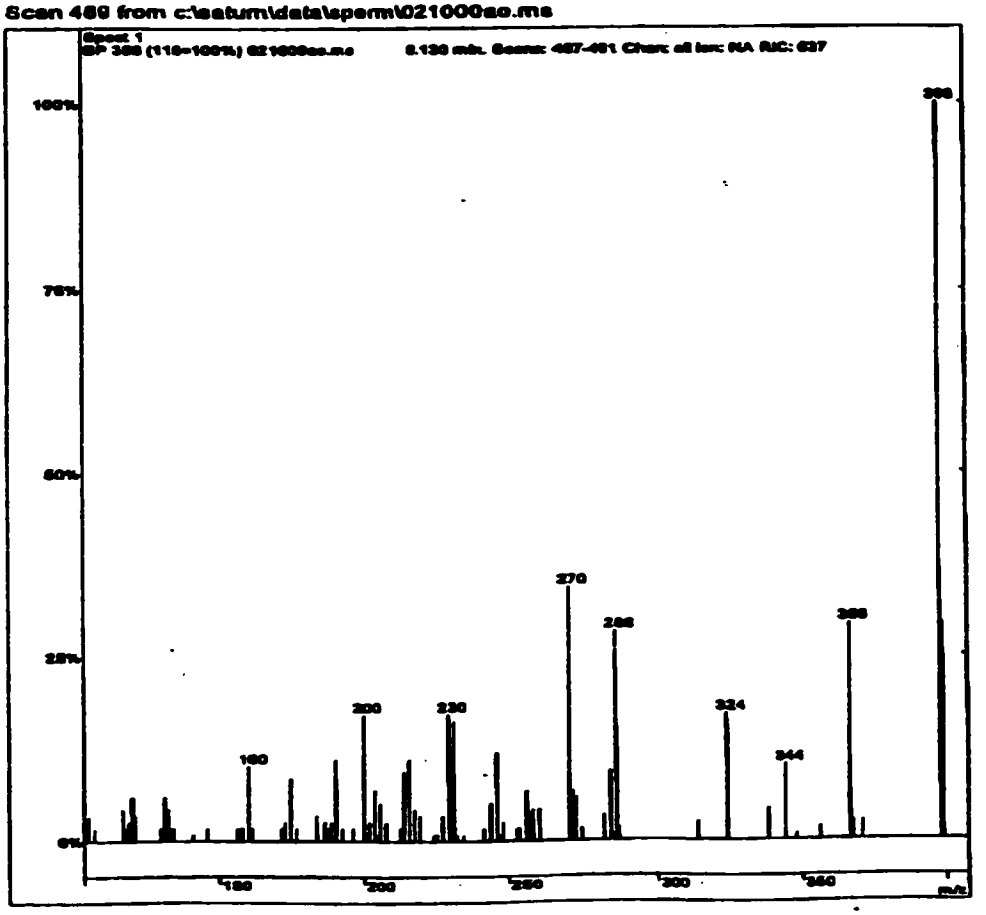
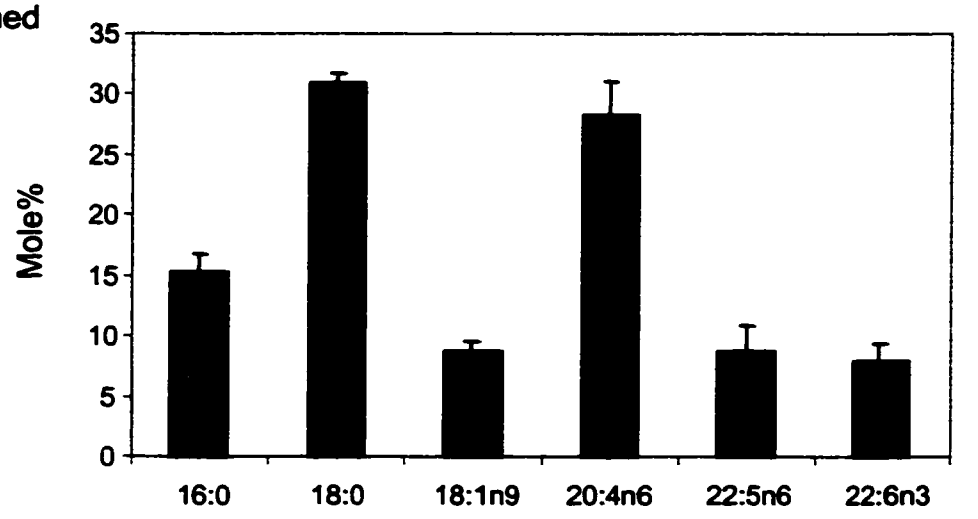
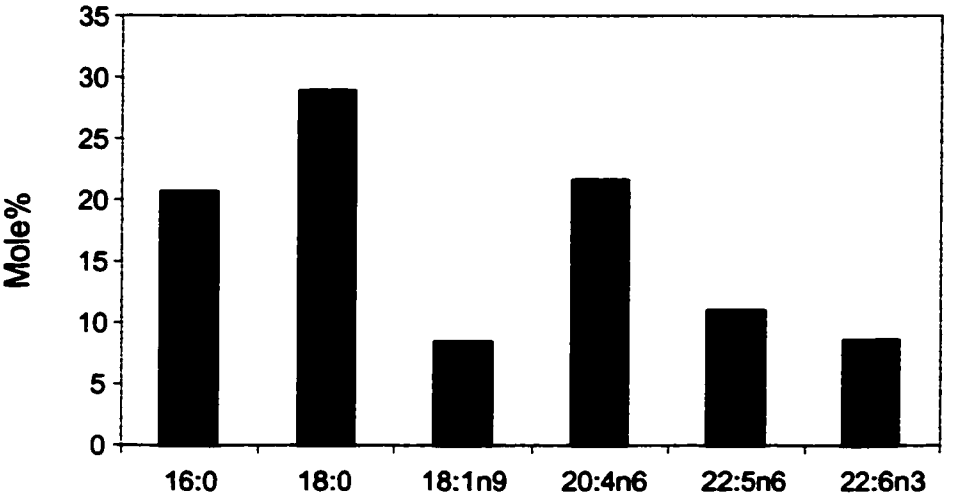


Figure 3.16: Molar distribution of the major PC-bound fatty acyl chains in WS (A), WCS (B), and PGCS (C). The distribution in (A) is expressed as mean ± standard error. The distributions in (B) and (C) are the average of two analyses.

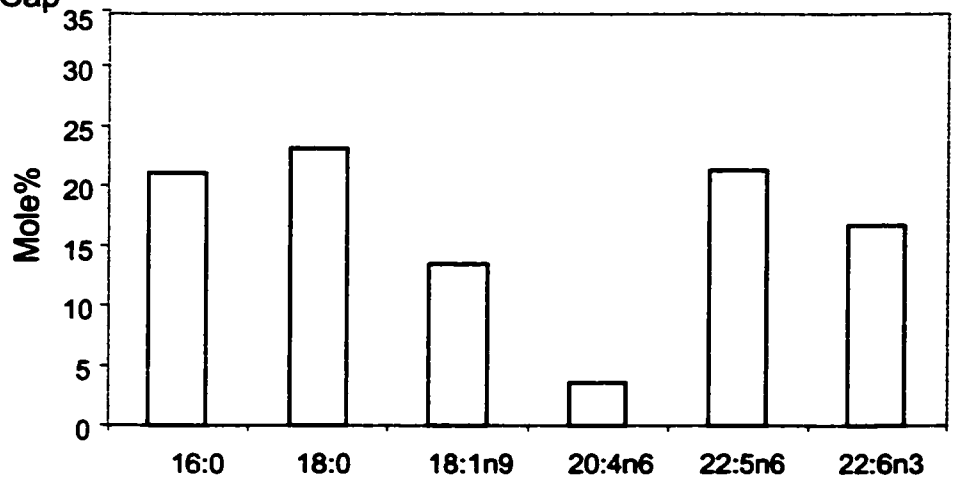
A) Washed



B) Washed Cap



C) PGC Cap



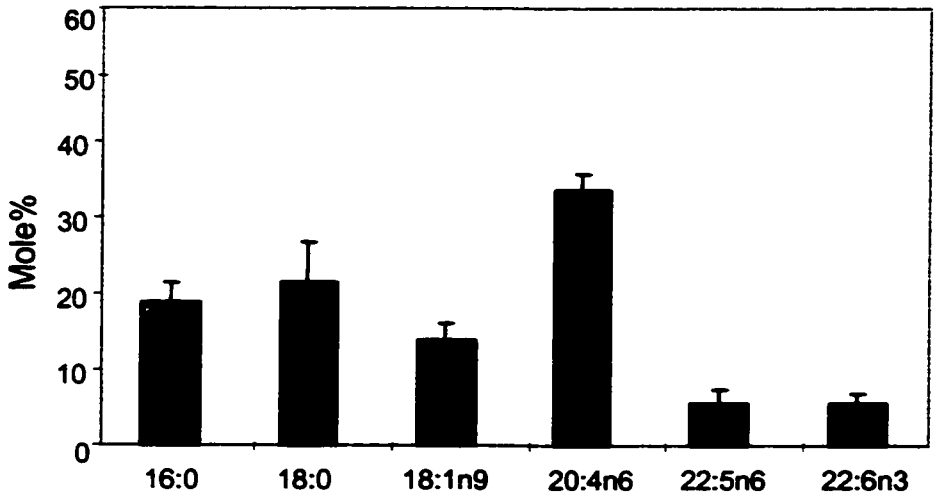
3.4.2 Molar Distribution of the Major PE-Bound Fatty Acyl Chains in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

In WS, the SFAs 16:0 and 18:0 constituted about 18 and 21 mole%, respectively (Figure 3.17A). About 13 mole% was 18:1n9 and the major PUFA was 20:4n6, which contributed about 33 mole%, while 22:5n6 and 22:6n3 were present at 5 mole% each. In WCS, the SFAs 16:0 and 18:0 constituted about 41 and 52 mole%, respectively (Figure 3.17B). About 5 mole% was 18:1n9, and 20:4n6 and 22:5n6 were not detected, while 22:6n3 was only present as 1 mole%. Small amounts of this sample were injected at the time of analysis, as shown by the low level of millivolts (Figure 3.12B), as compared to the other samples. Thus, if a small amount of sample was injected, the FAMEs that were present at lesser amounts, i.e., PUFAs, might not be detected. To confirm these results, i.e., that there were low levels of PUFAs, this sample was sent for analysis by GC-MS at Health Canada (Figure 3.14). The results obtained by GC-MS detected the same major FAMEs as those determined by GC, as well as 16:0 DMA (18%) and 18:0 DMA (2%) (Figure 3.14). The distribution of PE in WCS determined by GC-MS, not including the presence of 16:0 DMA or 18:0 DMA, was similar to the distribution determined by GC performed at the University of Guelph, and was used in place of these results.

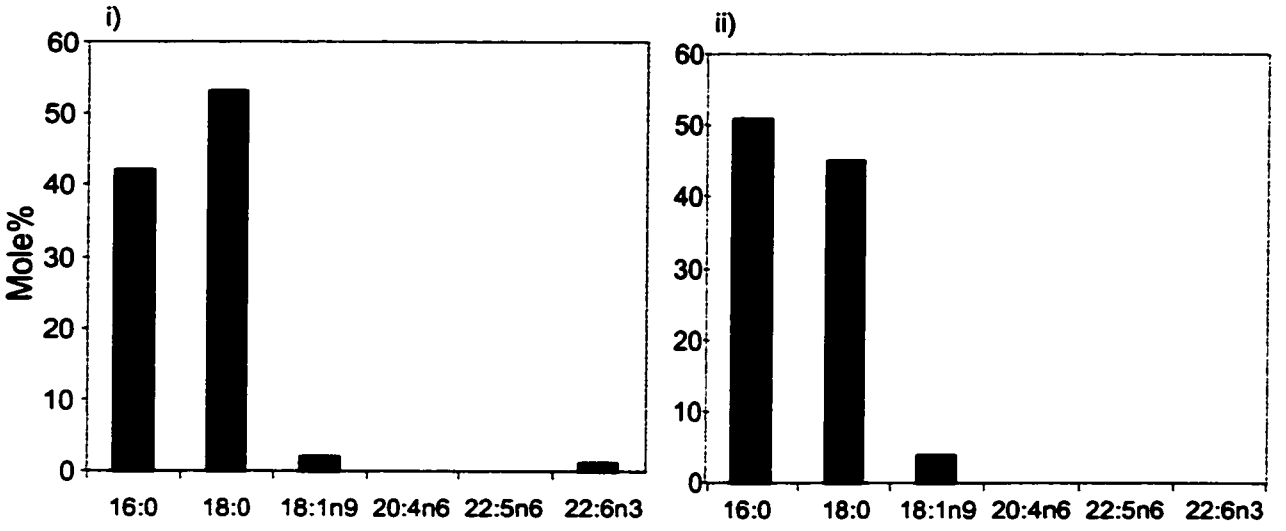
In PGCS, 16:0 and 18:0 constituted about 40 and 19 mole%, respectively (Figure 3.17C). The major unsaturated fatty acyl chain was 18:1n9 at about 24 mole%. About 8 mole% was 20:4n6, while 22:5n6 and 22:6n3 constituted about 5 and 4 mole%, respectively.

Figure 3.17: Molar distribution of the major PE-bound fatty acyl chains in WS (A), WCS (B) and PGCS (C). The distribution in (A) is expressed as mean ± standard error (n=3). For WCS (B), i) represents the molar distribution determined by GC at the University of Guelph and is the average of two analyses, while ii) represents the molar distribution determined by GC-MS at Health Canada. The distribution in (C) is the average of two analyses.

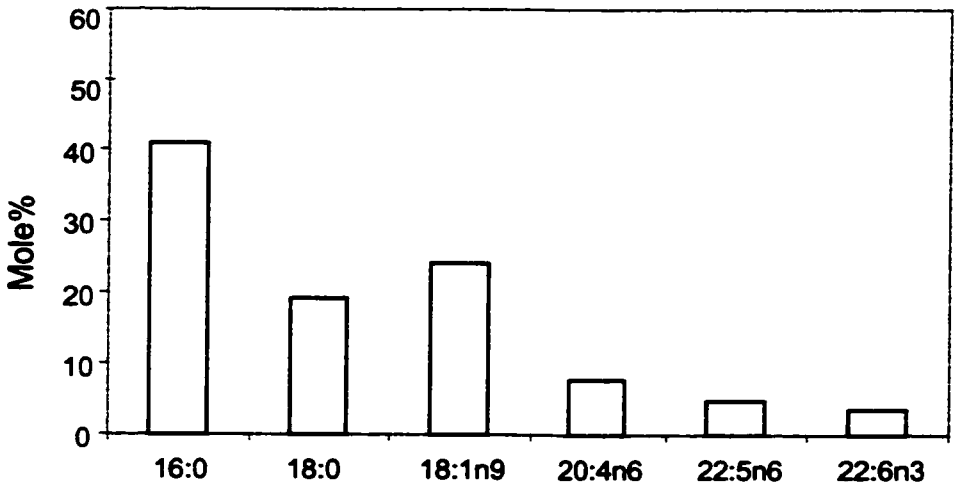
A) Washed



B) Washed Cap



C) PGC Cap

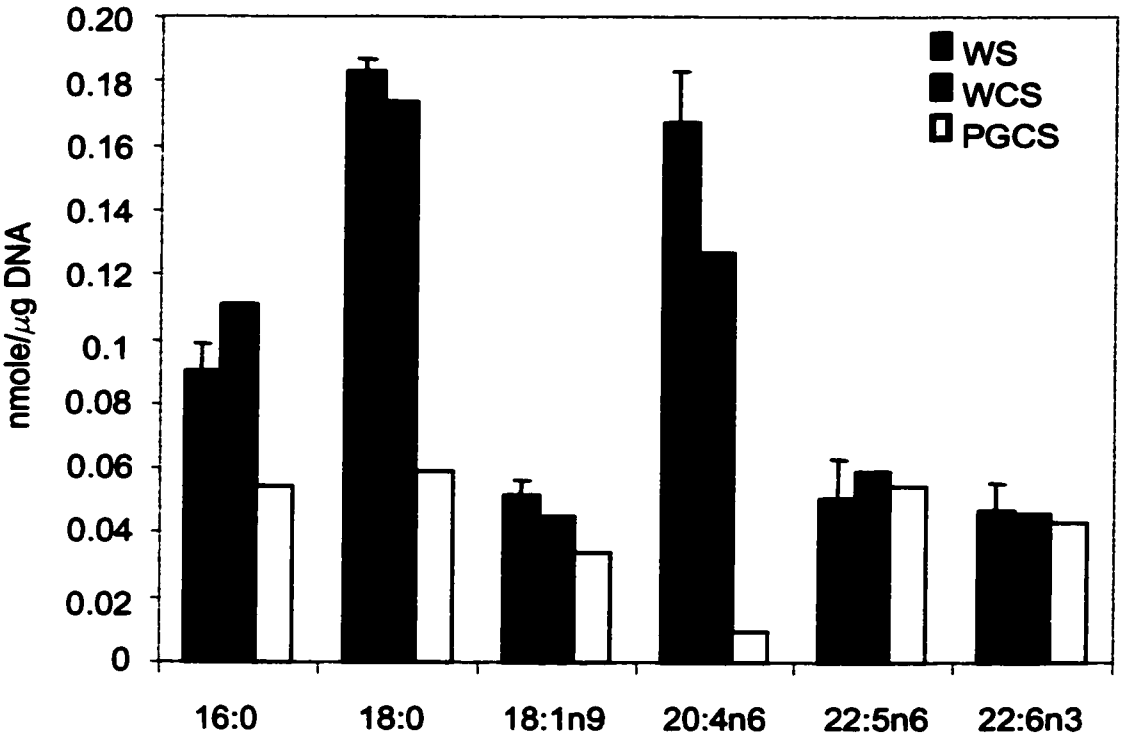


In summary, the molar distribution of the PE-bound SFAs was about double that in WCS compared to WS. All unsaturated fatty acyl chains were much lower, or not detected, following capacitation in the washed samples. In PGCS, the distribution of 16:0 was similar to the distribution in WCS, but the distribution of 18:0 was about half of that in the WCS. The distribution of the unsaturated fatty acyl chains was higher in the PGCS compared to the WCS, but were lower than the distribution seen in PC.

3.4.3 Levels of the Major PC-Bound Fatty Acyl Chains in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

Following capacitation in the washed sperm lipids, there was an increase in PC-bound 16:0 (0.090 nmol/μg DNA in WS vs. 0.111 nmol/μg DNA in WCS) and a decrease of PC-bound 20:4n6 (0.167 nmole/μg DNA in WS vs. 0.117 nmole/μg DNA in WCS) (Figure 3.18). In PGCS, all major FAMEs, except 20:4n6, were present at similar levels. The level of PC-bound 20:4n6 was much lower than the other FAMEs. PGCS had lower levels of PC-bound 16:0 and 18:0 compared to WCS, and while PC-bound 20:4n6 was the major PUFA in the WS, it was the minor PUFA in PGCS. The longer chain PUFAs, PC-bound 22:5n6 and 22:6n3, were present at similar levels in the WCS and PGCS.

Figure 3.18 Levels of the major PC-bound fatty acyl chains in WS, WCS and PGCS. The amount of each FAME was based on the level of PC in each sample (Table 3.1), and the molar distribution of each FAME (Figure 3.16 and Tables A.1, A.4 and A.7). The level of PC-bound FAMEs in WS is expressed as mean ± standard error (n=3), while the levels in WCS and PGCS are the average of two analyses.


0.20
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
nmole/μg DNA
WS
WCS
PGCS
16:0
18:0
18:1n9
20:4n6
22:5n6
22:6n3

3.4.4 Levels of the Major PE-Bound Fatty Acyl Chains in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

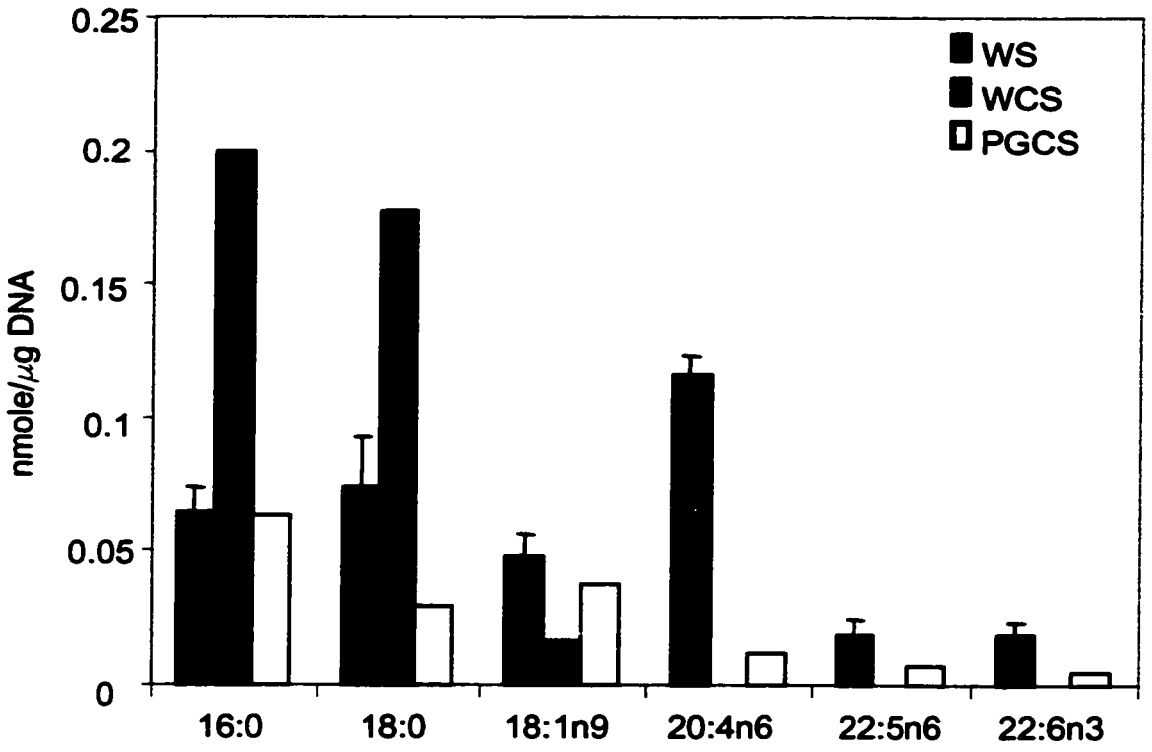
Following capacitation in the washed samples, there were large increases of PE-bound 16:0 and 18:0 (0.65 vs. 0.200 nmol/μg DNA and 0.074 vs. 0.177 nmol/μg DNA in WS and WCS, respectively) (Figure 3.19). There were also decreases of PE-bound 18:1n9, 20:4n6, 22:5n6 and 22:6n3 in the WCS compared to WS. In PGCS, PE-bound 16:0 and 18:0 were present at much lower levels than in WCS (Figure 3.19). The levels of PE-bound 18:1n9, 20:4n6 and 22:5n6 were higher in PGCS than in WCS.

3.5 Summary

No differences in the levels or molar distribution of cholesterol, PL or SGG were observed following capacitation in the washed samples (Figures 3.4 and 3.9). There was an increase of PC-bound 16:0 and a decrease of PC-bound 20:4n6 in WCS compared to WS. The most noticeable changes were observed in the PE-bound FAMEs following capacitation in the washed samples, in which there was an increase in 16:0 and 18:0, and a decrease of all unsaturated FAMEs, especially 20:4n6.

PGCS had significantly lower levels of cholesterol and PL compared to WC. Since cholesterol and PL were the major lipid components in sperm, their decreased levels resulted in the increased molar distribution of SGG, although the amount was similar to that of the washed samples. The PC-bound FAMEs of PGCS had lower levels of 16:0 and 18:0, with an increased molar distribution of PUFAs. The PE-bound FAMEs of PGCS had much lower levels of SFAs compared to WCS, with higher levels of unsaturated fatty acyl chains.

Figure 3.19: Levels of the major PE-bound fatty acyl chains in WS, WCS and PGCS. The amount of each FAME was calculated based on the level of PE in each sample (Table 3.1), and the molar distribution of each FAME (Figure 3.17 and Tables A.2, A.5 and A.8). The level of PE-bound FAMEs in WS is expressed as mean ± standard error (n=3), the level in WCS lipid represents one analysis, and the level in PGCS is the average of two analyses.


0.25
0.2
0.15
0.1
0.05
0
nmole/μg DNA
WS
WCS
PGCS
16:0
18:0
18:1n9
20:4n6
22:5n6
22:6n3

CHAPTER FOUR

DISCUSSION

4.1 Characterization of the Lipid Composition of Washed Non-Capacitated, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

To date, the only major change in lipid composition that has been described following capacitation in sperm from many species is a decrease in the cholesterol:PL molar ratio (Davis et al., 1979; Langlais and Roberts, 1985; Go and Wolf, 1985; Cross, 1998); however, no change was observed in mouse (Tanphaichitr et al., 1996). The lipid composition of human sperm prepared by PGC, a method which may partially capacitate the population of sperm that is selected based on the development of hyperactivated motility patterns, has been compared with washed sperm containing the whole population of cells, and lower levels of both cholesterol and PL were observed in PGC sperm (Sugkraroek et al., 1991). Characterization of the fatty acid composition in washed and PGC human sperm showed an increased molar distribution of 20:4n6 (5.5 vs. 10.5 mole%) and 22:6n3 (22.5 vs. 34.5 mole%) in PGC sperm (Lenzi et al., 1996). This suggests that there is increased membrane fluidity in the population of sperm having the higher fertilizing ability. This type of analysis, characterizing the fatty acid composition of sperm before and after capacitation, has not been performed in any other species.

The majority of the lipid characterization that has been performed to date has been determined in large animals (e.g., boar, bull, ram), from which a large number of sperm are obtained and provides enough lipid material for numerous analyses. Little information is known concerning the lipid composition of sperm from mice, even though the physiology and biochemistry of fertilization in this species has been widely

characterized. This lack of information about mouse sperm lipids is most likely the result of the large amount of material, and hence mice, that is required to perform these types of lipid analyses, and has resulted in a project that involved time consuming and laborious sample preparations. However, the results obtained from this study have provided new insight with respect to the lipid composition of sperm before and after capacitation, as well as the lipid composition of the sperm having higher fertilizing ability, i.e., PGC sperm.

4.2 Sperm-Egg Binding of Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

Percoll gradient centrifugation has been used as a method to separate cells or organelles in suspension based on differences in size or buoyant density (Pertoft, 2000) and many Percoll density gradients have been described for isolation of pure sperm cells that are viable and motile (Lessley and Garner, 1983; Dravland and Mortimer, 1985; Tanphaichitr et al., 1988; Lenzi et al., 2000). To prove that sperm prepared by PGC had a higher fertilizing ability than sperm prepared by conventional washing, a sperm-egg binding assay was performed comparing washed capacitated and PGC capacitated sperm. Significantly less washed capacitated sperm bound per egg than PGC capacitated sperm ($p < 0.0001$) (Figure 3.1A), and the washed capacitated sperm showed abnormal binding patterns, since they bound in clumps rather than uniformly around the eggs (Figure 3.1B). These results demonstrated that sperm prepared by PGC had a higher fertilizing ability than those prepared by conventional washing and were probably most representative of the sperm that are involved in fertilizing the egg. These results agreed with previous

work comparing human sperm prepared by PGC with sperm prepared by the swim-up method (Tanphaichitr et al., 1988). The swim up method involves carefully layering capacitating medium on the sperm pellet and allowing the motile, i.e., capacitated, sperm to swim up into the upper layer of medium, thus, a specific population of sperm is also selected using this method. However, conventional washing, which simply removed epididymal fluid from all populations of collected cells, was used in this study because of the number of sperm that were required to perform the analyses.

The difference in binding between washed capacitated and PGC capacitated sperm may have resulted for a couple of reasons. First, conventionally washed sperm consisted of all populations in which only a small percentage had high fertilizing ability. When performing this type of binding assay, it was possible that interference from the large percentage of sperm having low fertilizing ability may have reduced the ability of the fertile sperm to bind to the egg. The large number of sperm with low fertilizing ability had very low or no motility and may have formed barriers that the small percentage of motile sperm had to bypass to reach the egg. Since sperm prepared by PGC consisted of a population that was about 80 to 90% motile, this interference did not occur. Second, Percoll gradient centrifugation may be involved in stripping the sperm membrane of decapacitation factors, which would prepare the sperm for interacting with the ZP. Sperm prepared by conventional washing may not have shown this increased glycosylation pattern, and thus, their fertilizing ability was lower than that of PGC sperm. The higher motility and fertilizing ability of PGC capacitated sperm may also be determined in part by the lipid composition, such as the increased distributions of SGG (Section 4.3.2) and PUFAs (Section 4.5.1).

4.2.1 DNA Analysis

Sperm counting can be inaccurate and large errors may occur for a few reasons. First, sperm tend to stick to the surface of tubes, glass or plastic, over a short period of time (i.e., within 10 min), and thus, can result in a sperm count that is lower than the actual number of sperm present. In the DNA assay, sticking can be minimized by the addition of DTT to the sperm aliquot, which decondenses the sperm chromatin and, for an unclear reason, causes the sperm to lose their ability to adhere to the tube surface (Tanphaichitr et al., 1996). Also adding to the inconsistency of counting is intra-analysis variation and the necessary large-scale dilution of the sperm for counting. Thus, when the number of sperm (million per ml) is calculated based on the equation used (i.e., # of sperm in five squares x 2000 x dilution factor, pp. 35), all the sources of variations can result in a large error. This in turn can result in inaccuracies when data are expressed on a per sperm basis.

To overcome the inconsistencies experienced with sperm counting, sperm DNA levels were quantified to determine whether the amount of DNA per sperm cell was consistent. The two meiotic divisions of spermatogenic cells during spermatogenesis should result in each sperm containing one set of chromosomes and a DNA level that is one half of that in primary spermatocytes or somatic cells. However, meiosis can be abnormal, and result in aneuploidy and aberrancy in sperm DNA levels (Abruzzo and Hassold, 1995; Wyrobek et al., 1995). The sperm used for this study were collected from the vas deferens and caudal epididymis, and were assumed to be mature. In the case of washed sperm, which contained all populations, it might have been expected that some sperm were aneuploidic and/or contained aberrant amounts of DNA. Since PGC selected

for the sperm population that was mature and highly motile, it was expected that aneuploidy would not be present and that these sperm had normal chromosome numbers and DNA levels.

The amount of DNA remained consistent per sperm regardless of the preparation, i.e., about 4 pg DNA/sperm, with about 10% deviation (Figure 3.2B), and thus aberrant levels of DNA were not observed in the washed samples, as may have been expected. Therefore, it appeared that the levels of DNA per sperm in all three samples, whether prepared by conventional washing or PGC, were normal and consistent. These results were similar to, but slightly higher than what has been previously described in mouse sperm, i.e., 3.2 to 3.6 pg DNA/sperm (Pogany et al., 1981; Tanphaichitr et al, 1996). The scattering of DNA levels (Figure 3.2B) may reflect the inherent inaccuracy of cell counting; however, the levels of DNA in PGC sperm scattered over a smaller range than the levels in washed sperm (Figure 3.2B), and may suggest that PGC selected a more homogenous population of sperm having completely condensed chromatin. Since the amount of DNA per sperm was constant in all three preparations, and was more precise and consistent than sperm counting, the lipid data were expressed as per μg DNA instead of per sperm.

4.3 Characterization of the Levels of Cholesterol, PL, SGG, DAG and TAG in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

4.3.1 Levels of Cholesterol and Phospholipid

A decrease of cholesterol during capacitation in most species has long been accepted as a consequence of capacitation. This decrease occurs as a result of cholesterol

efflux from the sperm membrane following in vitro exposure to albumin (Langlais and Roberts, 1985; Go and Wolf, 1985; Cross, 1998) or HDL (Ehrenwald et al., 1990), both of which are present in oviductal fluid. More recently, β-cyclodextrins have been used (Choi and Toyoda, 1998; Iborra et al, 2000) because of their ability to specifically incorporate cholesterol into their core (Kilsdonk et al., 1995; Ohtani et al., 1989). Transfer of BSA-bound lipid to the sperm membrane has been observed when BSA was used as a capacitating agent (Davis et al., 1979; Go and Wolf, 1985), therefore the results obtained may not specifically represent changes of sperm plasma membrane lipids.

The removal of cholesterol may explain the increase in membrane fluidity that has been described to occur during capacitation (Wolf et al., 1986). Since cholesterol tends to condense lipids that are in a liquid crystalline phase, the phase occupied by the sperm membrane at physiological temperature (Palleschi and Silvestroni, 1996), its removal would reduce this condensing effect and thus, increase membrane fluidity. It has also been suggested that loss of cholesterol leads to exposure of a receptor for mannose on the sperm surface. Since mannose is located on the ZP and there has been evidence suggesting that cholesterol-enriched medium could prevent the expression of mannose receptors (Benoff, 1993; Benoff et al., 1993), removal of cholesterol could allow exposure of the mannose receptor on the sperm surface, preparing the sperm for ZP binding. There is also evidence indicating that cholesterol loss resulting from either BSA- or β-cyclodextrin-mediated capacitation caused an increase in tyrosine phosphorylation (Visconti et al, 1999; Osheroff et al., 1999), an event correlated with capacitation (Visconti et al., 1995; Visconti and Kopf, 1998).

Although a decrease of cholesterol was observed following capacitation in the washed samples (Figure 3.4), it was not found to be significant, which was consistent with previous work in mice (Tanphaichitr et al., 1996). This suggested that removal of cholesterol might not be a necessary event of capacitation in the mouse. There was also no change in the level of PL in the washed samples following in vitro incubation in BSA-containing capacitating medium (Figure 3.4).

The level of cholesterol in PGC capacitated sperm was significantly lower when compared to washed capacitated sperm ($p < 0.001$) (Figure 3.4) and may have been a result of cholesterol removal during PGC, cholesterol efflux during the 30 minute in vitro incubation in albumin-containing capacitating medium, or may be characteristic of the population of sperm that is selected for during PGC. Previous work in our lab has shown that there was no decrease in the level of cholesterol before or after a 30 minute incubation of PGC sperm in capacitating medium (Tanphaichitr et al., 1996). Therefore, the lower level is likely not a result of cholesterol efflux during incubation. It is difficult to prepare a non-capacitated PGC control, since the sperm pellet appears to be capacitated following centrifugation through a Percoll gradient, as evidenced by their hyper-activated motility patterns. In other words, the process of PGC may inevitably induce capacitation. Therefore, it was difficult to determine whether this decrease of cholesterol occurred as a result of capacitation.

The lower level of cholesterol observed in PGC sperm compared to washed sperm contrasted with previous work performed in this lab (Tanphaichitr et al., 1996), in which no change in the level of cholesterol was observed between washed and PGC sperm incubated with or without albumin-containing medium. Even though the same assay was

used, this difference may have occurred because of the purchase of a new spectrofluorometer. As a result, the assay protocol was modified and the sensitivity of the assay was increased about 2 times as it was adapted for a 96 well plate from a 1 ml cuvette. With the old assay protocol, it was possible to detect 0.1 μg cholesterol, while with the new assay protocol, it was possible to detect 0.05 μg cholesterol. Also, with the old machine, the fluorescence units of the highest point on the standard curve reached a maximum of about 20 and the values of each sample fluctuated ± two or three units, therefore, it was necessary to estimate the fluorescence of each sample within the range. With the new machine, the number of units of the highest point on the standard curve was about 20 times higher, which enabled a more accurate determination of the fluorescence of the sperm lipid samples.

The levels of PL were about 60% lower in the PGC capacitated sperm compared to the washed samples ($p < 0.001$), which was consistent with what has been previously reported (Tanphaichitr et al., 1996). This lower level of PL, along with the lower level of cholesterol, may have resulted from removal of lipid-containing membranous vesicles, which adhered to the surface of washed sperm (Tanphaichitr, 1988). These lipid-containing vesicles have been demonstrated to envelop the PM of washed human sperm, but not sperm prepared by PGC (Tanphaichitr, 1988). These membranous vesicles may be external to the sperm surface, with the possibility of being removed during PGC, or they can be part of the sperm, and therefore, non-removable. If they are external, their removal may partially explain the decreases in the levels of cholesterol and PL that were observed following PGC, as compared to the washed samples. There was also a possibility that the presence of these vesicles in washed sperm may have hindered the

action of albumin on the removal of cholesterol from the membrane of washed sperm. Therefore, the lack of these vesicles in PGC sperm may have allowed cholesterol to be more easily removed from the membrane. The lower PL levels may also have been characteristic of the sub-population of highly motile sperm that was selected for by PGC.

A decrease in the cholesterol:PL molar ratio, as a result of cholesterol removal, has been accepted as an important event of capacitation (Davis, 1981) and is necessary for the AR (Iborra et al., 2000). This causes a destabilization of the sperm membrane in preparation of the AR, in which the sperm PM fuses with the underlying outer acrosomal membrane. In this mouse model, there was an insignificant decrease in the cholesterol:PL molar ratio in the washed samples following capacitation, which is consistent with previously published results (Tanphaichitr et al., 1996). The cholesterol:PL molar ratio was also lower in PGC capacitated sperm compared to washed capacitated sperm, but this difference was not significant. Therefore, a decrease in the cholesterol:PL molar ratio may not be of great importance during capacitation in this mouse model, but other lipid changes, such as a remodelling of the PL-bound fatty acyl chains, may occur and play a role in increasing sperm membrane fluidity in preparation for the AR.

4.3.2 Levels of SGG

Lines of evidence have indicated the significance of SGG in sperm-egg interactions. Sperm pretreated with a polyclonal antibody monospecific to SGG lost their ability to bind to the egg ZP (White et al., 2000) and the egg PM (Ahnonkitpanit et al., 1999). Additionally, SGG liposomes were shown to bind directly to the egg ZP, while GG liposomes did not (White et al, 2000). These results suggest that the sulfate moiety

of SGG plays an important role in sperm-egg binding. In non-capacitated sperm, SGG has been shown to be highly concentrated in the apical ridge of the sperm head PM, while in capacitated sperm, the entire SGG fraction migrated towards the equatorial region of the sperm head PM (Gadella et al., 1994; Gadella et al., 1995), the part of the sperm that is important for binding and fusing to the egg PM. This result also implicates a role for SGG in sperm-egg interactions. SGG has also been shown to increase the rigidity of dimyristoylphosphatidylcholine model membranes (Attar et al., 2000). Therefore, while the fluidity of the sperm membrane may increase during capacitation as a result of a decreased cholesterol:PL molar ratio, SGG may be involved in giving the sperm membrane the sufficient rigidity required in the ZP/egg PM binding regions.

SGG has previously been shown to be stable during capacitation (Tanphaichitr et al., 1990) and was confirmed in this report by the unchanged level of SGG in washed and washed capacitated sperm lipids (Figure 3.4). In both washed samples, SGG constituted about 8 mole% of total lipids (Figure 3.9). The lower level of SGG in PGC capacitated sperm was not significant compared to the washed capacitated sperm, but it constituted about 14 mole% of the total lipids, as a result of the significantly lower levels of cholesterol and PL. PGC capacitated epididymal mouse sperm had a proven higher fertilizing ability, since they bound more sperm per egg compared to washed capacitated mouse sperm (Figure 3.1A). Therefore, the higher percentage of SGG in PGC capacitated mouse sperm may be positively correlated with their higher fertilizing ability.

4.3.3 Levels of DAG and TAG

In capacitated mouse sperm stimulated with calcium ionophore, progesterone or ZP, all of which are inducers of the AR, a Ca^{2+}-dependent increase in DAG was observed

(Roldan et al., 1994), suggesting that an increase in DAG levels is an important event and is required for signalling acrosomal exocytosis during the AR (Roldan, 1999). These increased levels have implicated DAG as a lipid second messenger by stimulating PLA_2 (Roldan and Fragio, 1994) and PKC (O'Toole et al., 1996). Generation of DAG during the AR has been shown to be a result of a Ca^{2+}-dependent activation of PI- and PC-specific PLC (Sheikhnejad and Srivastava, 1986; Roldan and Harrison, 1989; Roldan and Murase, 1994). Phospholipase D, which first generates phosphatidic acid that is converted to DAG by the action of phosphatidic acid phosphohydrolase, has not been demonstrated to be involved in signaling the AR (Roldan and Dawes, 1993; Roldan et al., 1994).

A great deal of variation accompanied the HPTLC method that was used to quantify the levels of DAG and TAG. For example, the intensity (i.e., number of pixels) of corresponding spots on different HPTLC plates was not consistent, as evidenced by the standard deviations of the DAG and TAG standard curves (Figure 3.8). This inconsistency may have resulted from the method used for staining and may have occurred because of variations in the concentrations of different Coomassie blue staining solutions, or variations in staining/destaining times, which could both affect the intensity of lipid staining. The amounts of DAG and TAG in the sperm samples were determined by comparing the pixel counts of these lipids with standard curves constructed from known amounts of DAG and TAG co-chromatographed on the same HPTLC plate. However, the range of pixels covered by the standard curves was small for both DAG and TAG (Figure 3.8), and thus, made it difficult to determine the amounts of these lipids in the sperm samples precisely. More work will be required to improve the consistency and

accuracy of this method for determining the levels of DAG and TAG.

Even though a great deal of variation accompanied this assay procedure, it did give an idea of the levels of DAG and TAG present in the sperm samples. Ultimately, the results obtained from this assay gave a better representation of the distribution of the different lipid classes in sperm, since cholesterol and PL are not the only lipid classes present in membranes. No change of DAG levels was observed among the three samples. Little attention has been paid to the role of TAG in sperm cells, most likely because it makes up a small percentage of the total lipids and may not be considered to be of importance. TAG generally functions as a storage site for fatty acyl chains (Gibbons et al., 2000). A significant decrease of TAG was observed in washed capacitated sperm compared to washed sperm ($p < 0.05$), which was seen visually on the HPTLC plate (Figure 3.7), and the level in PGC capacitated sperm was similar to that in washed capacitated sperm (Figure 3.4).

No functions for DAG and TAG during capacitation have been postulated in the literature. Although the role for DAG as a second messenger during the AR is well known (Roldan, 1999), it might serve a different purpose during capacitation. DAG and TAG may serve as a source of fatty acids for PL-bound fatty acyl chain remodelling during capacitation to help maintain membrane integrity (discussed in section 4.5.1), and thus the loss of their fatty acyl chains would result in decreased levels of these lipids following capacitation. This transfer of fatty acyl chains may involve a transacylase.

4.3.4 Summary

There were no significant differences between washed non-capacitated and washed capacitated sperm with respect to the levels or distribution of cholesterol, PL and

SGG. PGC capacitated sperm showed significantly lower levels of cholesterol and PL when compared to washed capacitated sperm. Since PGC sperm consisted of a population that was mainly motile having normal morphology and a higher fertilizing ability, this lipid composition might be characteristic of the specific population of sperm that is selected. Because it is not possible to prepare a non-capacitated PGC control, since PGC sperm appear to be partially capacitated following centrifugation through Percoll, it is difficult to differentiate between the changes that occur during capacitation and the lipid levels that are characteristic of the population of sperm selected for by PGC. Another possible explanation for the lower lipid levels in PGC capacitated sperm, as compared to those in washed sperm, is the absence of lipid-containing membranous vesicles, which were observed to envelop washed sperm, but not those prepared by PGC (Tanphaichitr et al., 1988). These vesicles probably consist of lipids as their main component and contribute to the higher levels measured in washed sperm. PGC may cause the removal of these vesicles, if they are external to the sperm surface, resulting in the lower lipid levels. Alternatively, if these vesicles are a part of the sperm, PGC may select for the population that does not contain these vesicles and thus, has a unique lipid composition.

4.4 Molar Distribution of the Major PL Classes

The major PL classes in washed non-capacitated, washed capacitated and PGC capacitated epididymal mouse sperm were determined by HPTLC and densitometric analysis to be PC and PE, with a minor contribution from SPH, which agrees with what is observed in other mammalian species (boar: Parks et al., 1987, Parks and Lynch, 1992,

Buhr et al., 1994; bull: Parks and Lynch, 1992, Hinkovska-Galcheva and Srivastava, 1993; human: Martinez and Morros, 1996; rabbit: Hinkovska-Galcheva and Srivastava, 1993; rat: Aveldano et al., 1992). There was no major difference in the distribution of these classes among the three samples. PL methylation has been described in golden hamster sperm during in vitro capacitation, with an increased methylation of 50% (Llanos and Meizel, 1983). Methylation would most likely occur to PE, resulting in generation of methyl PE, dimethyl PE and PC, and may be involved in increasing membrane fluidity, since the head groups of these generated PLs occupy more space than PE and interact less with neighboring PLs as a result of shielding of the amine positive charge by the methyl group(s). An increase of PC or methyl PE was not observed in our mouse model, and thus PL methylation did not appear to be an important event during mouse sperm capacitation.

No PS or PI was detected, although the presence of these PLs has been described in sperm cells from a variety of species. Nonetheless, they tend to make up less than 5 mole% of total PLs (Evans et al., 1980; Aveldano et al., 1992; Parks and Lynch, 1992). It is likely that PS and PI were present in these mouse sperm lipid preparations; however, they were not detected, probably because the amount of lipid used was not enough to visualize these sub-classes using this method. With Coomassie blue staining, it was possible to detect a minimum of about 2 μg of each PL class (approximate amount of SPH in each sample based on percent distribution and amount of lipids applied to HPTLC plate). Thus, it appears that PS and PI were present at less than 4% of total PLs in these samples.

The role of specific PL classes has been studied to determine their effects on

capacitation and the AR. LPC, because of its non-bilayer forming structure, has been successfully used to induce the AR (Yanagimachi and Suzuki, 1985; Llanos et al., 1993; de Lamirande and Gagnon, 1995; Riffo and Parraga, 1996); however, the presence of LPC, or LPE, was not detected in the washed or PGC sperm lipids, as analyzed by HPTLC. Incubation in medium supplemented with PC increased acrosomal responsiveness compared to controls (Cross, 1994, 1996), while the role of SPH was postulated to influence the rate of capacitation by slowing the loss of cholesterol from the sperm membrane (Cross, 2000), possibly because of its preferential interaction with cholesterol (Brown and London, 2000). We were not able to detect any changes in the PL class distribution that would suggest any involvement in capacitation in this mouse model.

With orcinol/acid charring, a few blue-staining spots were detected, which suggested the presence of sugar-containing lipids (Figure 3.10A). SGG was present in these samples; however, it migrated quite high on the HPTLC plate in the solvent system used and could not be differentiated from cholesterol and neutral lipids. These uncharacterized glycolipid spots could be gangliosides or other glycosphingolipids, such as SGC, GM_1, GM_3, GD_{1a}, and GD_{1b}, which have been described in sperm of various species (Levine et al., 1976; Mack et al., 1986, 1987; Ritter et al., 1987; Ohta et al., 1999). SGC and GM_1 were tested by HPTLC in an attempt to characterize these unknown lipid spots. Both glycolipids stained blue with orcinol/acid charring, indicating the presence of sugar moieties, but their R_f's did not correspond with any of the blue spots in the samples.

4.5 Characterization of the PC- and PE-Bound FAMEs in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

The FAME analysis of washed sperm was performed three times. Washed capacitated sperm lipids were analysed three times, but one analysis was very inconsistent with the other two and was discarded. The main source of inconsistencies with washed capacitated sperm was obtained with PE, more specifically with the PUFAs, and may have been a result of the sensitivity limit of the GC such that if the amounts of lipid injected for each FAME analysis was too low, then the minor components may not have been detected. It is likely that, for this reason, 20:4n6, 22:5n6 and 22:6n3 were not detectable in one of the analyses (Table A.5). Subsequent analysis by GC-MS confirmed that PUFAs were present at low levels in PE of washed capacitated sperm (Figure 3.14; Table A.5). About 80 mice were required to have enough lipid material to generate FAMEs for analysis of PGC sperm, therefore, this sample was repeated twice with a maximum deviation of about ± 20%.

Sperm, including those from mice, have been shown to contain a high proportion of plasmalogens (Poulos et al., 1973; Nikolopoulou et al., 1985; Alvarez et al., 1985; Parks and Lynch, 1992; Lin et al., 1993; Brouwers et al., 1998). Acid methanolysis of a plasmalogen yields a DMA (from the alk-1-enylether bond at the *sn*-1 position) and a FAME (from the ester linkage at the *sn*-2 position). The GC analysis performed at the University of Guelph analysed only FAMEs and did not give consideration to the presence of potentially abundant DMAs. Therefore, if plasmalogens were present in these lipid samples, the FAME generated from the *sn*-2 position was analysed along with the FAMEs generated from the diacyl PLs, while the DMA generated from the *sn*-1

position was not. Since the *sn*-1 position is generally occupied by either a saturated or monounsaturated fatty acid/alcohol (depending in the type of linkage), if plasmalogens did in fact make up a high proportion of the PLs in these mouse sperm lipids, it would be expected that the composition of the fatty alcohol present in plasmalogens would be 16:0, 18:0 and 18:1n9. Characterization of the levels of DMAs in these samples is ongoing in our laboratory in collaboration with Dr. Renaud Vincent at Health Canada (Ottawa, ON).

4.5.1 Characterization of the Major PC-Bound Fatty Acyl Chains in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

Following capacitation in the washed samples, there appeared to be an increase in PC-bound 16:0 (Figure 3.18), while PC-bound 20:4n6 decreased by about 25% following capacitation. This decrease of 20:4n6 suggested the possible activation of PLA_2, since this enzyme is responsible for hydrolysing the *sn*-2 ester bond of PLs resulting in the release of fatty acids, which are generally PUFAs. This enzyme may also show specificity for 20:4n6. The other products of PLA_2 activity are lysophospholipids, which have shown involvement in the AR, but may also play a role in capacitation.

PLA_2's make up a large superfamily of enzymes that serve a significant role in PL metabolism, membrane remodelling, signal transduction and eicosanoid biosynthesis (Dennis, 1994). They are divided into nine groups based on sequence information (Dennis, 1994, 1997). Generally, they are either extracellular, low molecular weight secretory enzymes (13-18 kD), which require millimolar levels of Ca^{2+} for activation (Groups I, II, III, V, VII and IX), or they are larger, intracellular cytosolic enzymes (85 kD), which require either no or micromolar levels of Ca^{2+} (Groups IV, VI, VIII) (Serhan et al., 1996; Dennis, 1997). The cytoplasmic PLA_2's also show high selectivity for

20:4n6 or PUFAs (Channon and Leslie, 1990; Kramer et al., 1991; Diez et al., 1992; Diez et al., 1994). In addition to Ca^{2+}, serine phosphorylation has been shown to be involved in regulating cytoplasmic PLA_2 activity (Xing and Mattera, 1992; Nemenoff et al., 1993).

PLA_2 has been described in sperm of different species (human: Fry et al., 1992; boar: Roldan and Vazquez, 1996; hamster: Riffo and Parraga, 1997). There is little information available concerning which PLA_2 group(s) is present, but partial characterization has revealed the presence of a 14 to 16 kD PLA_2 (Langlais et al., 1992), suggesting a member of the secretory PLA_2s. However, the protocols of acid extraction that have been used have reduced the possibility of isolating high molecular weight isozymes that might be present (Roldan, 1999). Its activity has generally been associated with the AR, since it requires millimolar levels of Ca^{2+} that are present during the AR (Roldan and Mollinedo, 1991; Roldan, 1999). It has also been localized to the acrosomal region (Riffo et al., 1992), and it is activated in human (Baldi et al., 1993) and boar spermatozoa (Roldan and Vazquez, 1996) in response to progesterone, an inducer of the AR. The action of PLA_2 during the AR results in the generation of fatty acids and lysophospholipids (e.g., LPC and LPE), the major role of which appears to be related to membrane perturbation resulting in fusion of the PM with the underlying outer acrosomal membrane (Yanagimachi, 1994). Generation of LPC or LPE was not observed in washed or PGC mouse sperm lipids following capacitation. Increased levels of lysophospholipids during capacitation may not be favourable for the sperm membrane because it may cause premature AR; therefore, if PLA_2 is activated during capacitation, it may be different from the one involved in the AR and may play a role that is specific for capacitation. For example, PL-bound fatty acyl chain remodelling may occur by a PLA_2

containing transacylase activity, as described in murine P388D$_1$ (Lio and Dennis, 1998; Balisinde et al., 1995) and CHO cells (Lio and Dennis, 1998), which is most evident in PE (Fig. 3.19). Activation of PLA_2 during capacitation may also be involved in the release of 20:4n6, since this fatty acid was seen to decrease in both PC and PE (Figs. 3.18 and 3.19), and has shown a potential involvement in stimulating the AR. These two possible roles of PLA_2 are addressed later on.

A small nanomolar increase in Ca^{2+} has been observed during capacitation in mouse sperm (Singh et al., 1978; Ruknudin and Silver, 1990); therefore, it is possible that the PLA_2 that appears to be specific for 20:4n6 during capacitation, is a higher molecular weight enzyme that requires no, or a small amount of Ca^{2+} for activation (Clark et al., 1995; Serhan et al., 1996). A Ca^{2+}-independent PLA_2 isolated from murine P388D$_1$ macrophages and Chinese hamster ovary cells has demonstrated lysophospholipase and transacylase activity (Lio and Dennis, 1998) and may have characteristics that are also suited to the situation in mouse sperm. Many different types of PLA_2s have been isolated from a variety of tissues, many demonstrating unique properties and activities. Therefore, if PLA_2 is activated during capacitation in sperm, it may also be specific and possess unique properties to facilitate the modifications that were observed in this mouse model.

An important role appears to be played by 20:4n6 in sperm, since it has been demonstrated to induce or accelerate the AR (Meizel and Turner, 1984) via the action of prostaglandins (Meizel and Turner, 1984; Roy and Ratnam, 1992; Shalev et al., 1994). Therefore, the possible activation of PLA_2 during capacitation may be necessary for the release of 20:4n6 that is further metabolized to prostaglandins, which may be involved in

stimulating the AR.

It is interesting to note that there was no decrease in PC-bound 22:5n6 or 22:6n3 following capacitation in the washed samples (Figure 3.18). There is no evidence about how 22:6n3-containing PLs are degraded by PLA_2. A Ca^{2+}-independent cytosolic PLA_2 isolated from rabbit platelets has been described that can discriminate between PE-bound 20:4n6 and 22:6n3 and preferentially hydrolyses 20:4n6 (Shikano et al., 1994). Although this enzyme was only tested using PE, it may have a similar effect on PC. An increase of PC-bound 16:0 was observed, and may suggest that a PLA_2 demonstrating transacylase activity, such as the one described above (Lio and Dennis, 1998), may be of significance in sperm.

A release of PC-bound 20:4n6 results in the generation of LPC, which is a membrane perturbing lipid that has been used to stimulate the AR in sperm by destabilizing the membrane and causing fusion of the plasma membrane with the underlying outer acrosomal membrane (Yanagimachi and Suzuki, 1985; Llanos et al., 1993; de Lamirande and Gagnon, 1995; Riffo and Parraga, 1996). During capacitation, this would not be a favourable event since it might lead to premature AR before reaching the vicinity of the egg and thus, reduce the chances of fertilization. LPC was not detected following capacitation in the washed samples, but the increase in PC-bound 16:0 suggests that immediate incorporation of this fatty acid occurred to maintain the integrity of the membrane. Possible sources of 16:0 fatty acids include BSA, since it is known to contain PLs having saturated fatty acyl chains (Davis et al., 1979; Go and Wolf, 1985). Transfer of PL from albumin to the sperm membrane has been observed during in vitro incubation (Davis et al., 1979; Go and Wolf, 1985). In turn, this transferred PL may be acted on by

a PLA_2 containing transacylase activity (Yamashita et al., 1997; Lio and Dennis, 1998), which could transfer SFA from the incorporated PL to sperm membrane LPC (Davis et al., 1979). If PL was transferred from BSA to the sperm membrane, it would be expected that the level of PL would be higher in washed capacitated sperm compared to washed sperm. However, no change in PL levels was observed following incubation with BSA, therefore, transfer of PL from BSA may not occur and 16:0 may be coming from somewhere else. Other possible sources could be DAG and TAG, whose fatty acyl chains may also be a substrate for transacylase activity.

When comparing the washed capacitated and PGC capacitated sperm, although the molar distribution of SFAs was similar in the two samples (Figure 3.16), the amounts were quite different (Figure 3.18). Since the level of PL in PGC capacitated sperm was about 60% lower compared to washed capacitated sperm (Figure 3.4), the fact that the amount of PC-bound 16:0 and 18:0 in PGC capacitated sperm was less than half of the amount in washed capacitated sperm was as expected. However, the most drastic difference occurred with PC-bound 20:4n6, which was present at about 90% lower levels in PGC capacitated sperm compared to washed capacitated sperm. This suggested that low levels of 20:4n6 were an important characteristic of the population of sperm having higher fertilizing ability, and may further implicate the activation of PLA_2 during capacitation. However, the low level could also be characteristic of the population of sperm that is selected for during PGC. Since 20:4n6 has been shown to increase the incidence of AR (Meizel and Turner, 1984), it may be more favourable for 20:4n6 levels to be initially low because any factor that could result in its release would stimulate premature AR. It was also interesting that, although the levels of 22:5n6 and 22:6n3 in

PGC capacitated sperm were similar to those in washed capacitated sperm, the molar distribution of these longer chain PUFAs was higher in PGC capacitated sperm compared to the washed samples (Figure 3.16), which suggested that the membrane fluidity is higher in the population of sperm prepared by PGC, and may be positively correlated with the higher motility and fertilizing ability of PGC capacitated sperm (Figure 3.1A).

4.5.2 Characterization of the Major PE-Bound Fatty Acyl Chains in Washed, Washed Capacitated and PGC Capacitated Epididymal Mouse Sperm

Increases in the molar distribution and levels of PE-bound 16:0 and 18:0 following capacitation were observed in the washed samples, along with decreases of 18:1n9, 20:4n6, 22:5n6 and 22:6n3 (Figure 3.17 and 3.19). These changes further support the idea of PLA_2 activation during capacitation for the purpose of remodelling the PL-bound fatty acyl chain composition, and appears to be more specific for PE than for PC. Unlike the specificity for a decrease of 20:4n6 observed in PC, decreases of all unsaturated fatty acids were observed in PE. The action of PLA_2 on PE results in the generation of LPE, and, as in the case of LPC, may not be a favourable situation for the sperm membrane during capacitation, since LPE is also a membrane perturbing lipid that can cause non-bilayer structures (Tilcock et al., 1986). Therefore, the increased levels of PE-bound 16:0 and 18:0 in washed capacitated sperm may not be surprising, and may be involved in re-generating diacyl PE that would maintain the integrity of the sperm membrane until sperm cells reach the ZP. As postulated for PC, the source of 16:0 and 18:0 fatty acids may be BSA-bound PLs, DAG or TAG, and may be immediately transferred to LPE by a PLA_2 that also possesses transacylase activity. The unsaturated

fatty acids that are released may be further metabolized to products that can be utilized to stimulate the AR, i.e., the generation of prostaglandins from 20:4n6.

The levels of all PE-bound fatty acyl chains in PGC capacitated sperm were low. The levels of 20:4n6 may have been low for the same reasons that the levels were low in PC, i.e., activation of PLA_2 occurs during capacitation resulting in the release of 20:4n6 which is further metabolized to other products (i.e., prostaglandins). Alternatively, PGC may select a population of sperm already containing low levels of PE-bound 20:4n6.

4.5.3 Summary

Only minor differences existed between the PC-bound fatty acyl chain composition of washed non-capacitated and washed capacitated epididymal mouse sperm. These differences included an increase of 16:0 and a decrease of 20:4n6. This suggested the activation of PLA_2 during capacitation, resulting in the release of 20:4n6, with subsequent transacylation of 16:0, possibly from the fatty acyl chains of BSA-bound PLs that were incorporated in to the membrane during incubation, or from DAG or TAG already present, by the action of a transacylase activity of the PLA_2. PE showed significant differences in the fatty acyl chain composition in washed capacitated sperm compared to washed sperm. Following capacitation, there were major decreases of all unsaturated fatty acyl chains, especially 20:4n6, which has demonstrated an involvement in stimulating the AR (Meizel and Turner, 1984; Roy and Ratnam, 1992; Shalev et al., 1994). Large increases in 16:0 and 18:0 were also observed, further suggesting the presence of a transacylase. Since the generation of lysophospholipids was not observed following capacitation, this transacylase activity might immediately transfer a SFA to the

lysophospholipid generated by the action of PLA_2 and help maintain the integrity of the membrane to reduce the possibility of premature AR.

The activation of PLA_2 during capacitation would result in the generation of lysophospholipids, along with the release of the fatty acyl chains at the *sn*-2 position. These lysophospholipids form an unfavorable membrane structure since they can form non-bilayer structures that could destabilize the membrane. Although membrane destabilization is a favorable event during the AR, its development during capacitation could result in premature AR and thus, reduced fertilizing ability of the sperm. It is possible that as fatty acids were hydrolyzed from PC and PE by PLA_2 activity, transacylation of SFAs to the generated lysophospholipids occurred immediately in order to maintain the integrity of the membrane. The potential source of fatty acids for transacylation could be BSA-bound PLs, or sperm membrane DAG and TAG present in the sperm membrane.

Sperm prepared by PGC were enriched in the PC-bound long chain PUFAs 22:5n6 and 22:6n3. Since PC was the major PL class in these sperm samples, this suggested that increased membrane fluidity was characteristic of the population of sperm selected for by PGC, which had higher motility and fertilizing ability (Figure 3.1A). The lower levels of 20:4n6 in PGC capacitated sperm compared to washed capacitated sperm suggested two things: either 20:4n6 was decreased during capacitation as a result of activation of PLA_2, or low levels of 20:4n6 were characteristic of sperm prepared by PGC. Since a non-capacitated PGC control was not prepared, it was difficult to make a definite conclusion as to why the levels of 20:4n6 were lower in PGC sperm compared to washed sperm.

4.6 Significance of Research Findings

In summary, the present report characterizes for the first time, the changes in lipid class composition, and the PC- and PE-bound fatty acyl chain levels and distribution in mouse sperm following in vitro capacitation. The only major difference observed in the lipid class distribution following capacitation in the washed sperm was a decrease of TAG levels. Major changes were observed in the fatty acyl chain compositions of PC and PE following capacitation. In vitro incubation of washed sperm in capacitating medium resulted in a decrease of both PC- and PE-bound 20:4n6, suggesting the activation of PLA_2. The released 20:4n6 may be further metabolized to prostaglandins, which have been shown to increase the acrosomal responsiveness of sperm (Meizel and Turner, 1984; Roy and Ratnam, 1992; Shalev et al., 1994).

This report also characterizes the lipid class, and PC- and PE-bound fatty acyl chain compositions in PGC capacitated sperm for the first time. Capacitated sperm prepared by PGC bound more sperm per egg than washed capacitated sperm, indicating that PGC capacitated sperm had a higher fertilizing ability. This higher fertilizing ability may have been partially determined by the lipids of PGC capacitated sperm, since they demonstrated a unique lipid composition compared to the washed samples. The levels of cholesterol and PL were significantly lower in the washed samples and may have resulted from the removal of lipid-containing membranous vesicles that have been shown to envelope sperm prepared by conventional washing, but not by PGC (Tanphaichitr et al., 1988). No difference in the level of SGG was observed between washed and PGC sperm lipids; however, the molar distribution of SGG was about two times higher in PGC capacitated sperm. Since SGG has been implicated to play a role in sperm-egg

interaction (Ahnonkitpanit et al., 1999; White et al., 2000), its increased molar distribution in PGC capacitated sperm may attribute to the enhanced fertilizing ability of these sperm. SGG may be directly involved in interacting with the egg, while also helping to maintain rigid domains in the fluid sperm membrane that are involved in sperm-egg binding. The PC- and PE-bound fatty acyl chain composition of PGC capacitated sperm was also unique, as compared to that of washed capacitated sperm, and included an increased molar distribution of the PC-bound PUFAs 22:5n6 and 22:6n3, which suggested that increased membrane fluidity may be of importance in fertilizing ability. Low levels of PC- and PE-bound 20:4n6 were observed in PGC capacitated sperm, but since we could not prepare a non-capacitated PGC control, it was not possible to determine whether these low levels were a result of possible PLA_2 activity during capacitation, or whether they were characteristic of the population of sperm that was selected for by PGC.

The results from this study confirm that sperm prepared by PGC have a higher fertilizing ability than sperm prepared by conventional washing, as demonstrated by their greater ability to bind to ZP. The higher motility and fertilizing ability of PGC capacitated sperm compared to washed capacitated sperm, may be partially determined by their lipid composition, i.e., low levels of cholesterol and PL, along with an increased molar distribution of SGG and the long chain PUFAs 22:5n6 and 22:6n3. Therefore, sperm prepared by PGC should be used for fertilization studies, since they may provide a better representation of the sperm involved in sperm-egg binding.

Further characterization of the presence of DMAs is ongoing in our laboratory and will give information concerning the levels of plasmalogens in mouse sperm

prepared by conventional washing and PGC. Although the function of plasmalogens in sperm is not clearly understood, it will be interesting to determine whether any changes in the levels of this PL sub-class occur following capacitation in the washed samples, and whether there is a difference in the level of plasmalogens in PGC sperm compared to that of washed sperm.

This study has provided a prototype in correlating sperm fertilizing ability with membrane lipid composition, and can be applied to other species. Specifically, our analysis has revealed information concerning the lipid composition of sperm as they prepare for fertilization, and may be useful in developing suitable methods for cryopreservation of sperm from different species for long-term storage. Cryopreservation of sperm has become an important field of study for many reasons, including management of infertility, maintenance of populations of agricultural and endangered species, and preservation of transgenic animal strains. Cryopreserved sperm can also be made available for immediate use and will facilitate research related to fertilization, embryo development and transgenic mouse production. For cryopreservation to be successful, formation of intracellular ice during cooling must be avoided, and is achieved by the use of cryoprotectants such as dimethylsulfoxide and glycerol (Miyamoto and Ishibashi, 1978; McGann and Walterson, 1987), which may act by replacing intracellular water and do not form crystals when frozen. Sperm are also commonly frozen in the presence of TEST-yolk buffer, which contains egg yolk lipids that are composed mainly of lipid-bound saturated fatty acyl chains and cholesterol (Songsasen and Leibo, 1997), and thus may act to help stabilize the sperm membrane during freezing.

Membrane lipid dynamics of sperm appear to be affected by the freezing and storage processes involved in cryopreservation, since the PM of the acrosome and postacrosome of cryopreserved human sperm was found to be more rigid than that of fresh sperm (James et al., 1999). Sperm may be especially affected during cryopreservation because of their high degree of unsaturation, which makes them prone to oxidation, and low levels of cholesterol (White, 1993). Varying degrees of success with the cryopreservation process have been obtained depending on the species used and may be partially explained by the differences in lipid composition among species (White, 1993). Therefore, it may be beneficial to alter the sperm membrane composition so that it stable enough to withstand the stresses involved with cryopreservation and can help to maintain the fertilizing ability of the sperm post-thaw. Since lipids are the major component of the cell membrane, modifying the lipid composition for cryopreservation may make it possible to maintain the integrity of the sperm membrane. This may involve freezing sperm in the presence of a cryoprotectant such as dimethylsulfoxide or glycerol to minimize intracellular ice crystal formation, as well as a lipid-enriched solution to alter the lipid composition of the sperm membrane.

The first step in designing a lipid-enriched solution for sperm from a given species would be to select an appropriate population of sperm, characterize its lipid composition and then determine how that composition can be modified to protect the cells from cold shock and help to maintain or possibly increase post-thaw fertilizing ability. Post-thaw sperm quality was increased when the most motile human sperm (selected by the swim-up method) were used for freezing (Esteves et al., 2000). Since

sperm prepared by PGC have a higher fertilizing ability, using this population of sperm would most likely give the most successful results post-thaw.

When designing a lipid-enriched freezing solution, the observation that sperm from mammals containing equal proportions of cholesterol and PL, such as human sperm (Poulos and White, 1973), may be less susceptible to cold shock than those with a lower cholesterol:PL molar ratio (Drobnis et al., 1993; White, 1993) may be important. The increased level of cholesterol may help to stabilize the membrane during cooling (Drobnis et al., 1993; White, 1993). Therefore, addition of cholesterol to bring the cholesterol:PL molar ratio closer to one may increase the stability of the sperm membrane during cryopreservation. This could be particularly important in the case of PGC sperm from mice, since they possess a cholesterol:PL molar ratio of 0.29, as shown by this study.

Another lipid molecule that may be involved in stabilizing the sperm membrane is SGG. Spectroscopic analysis of a non-sulfated analog of SGG, GC, indicates that the temperature and time induced formation of the lamellar crystalline structure was accompanied by partial dehydration (Bou Khalil et al., 2001). Thus, addition of excess SGG, a germ cell specific sulfoglycolipid that is present as 8 mole% in washed sperm and 14 mole% in PGC sperm (Figure 3.9), may help to prevent water molecules from entering the cell by interacting with them through their sulfated head group. Therefore, increasing the distribution of SGG to a certain level and may help to minimize entry of water into the cell via a dehydration effect. The increased distribution of SGG may also increase the fertilizing ability of the sperm post-thaw.

Finally, the enrichment of PUFAs in sperm suggests that they may be at higher risk of being oxidized (Cunnane, 1994; Gardner, 1989). To prevent oxidation of unsaturated fatty acyl chains, which appear to play an important role in sperm membrane fluidity, it may be beneficial to include antioxidants in the lipid-enriched solution. The role of plasmalogens in sperm is not clearly understood; however, it may also be beneficial to include them because of their potential antioxidant function (Engelmann et al., 1994; Zoeller et al., 1999).

In conclusion, to increase the survival rate and fertilizing ability of sperm following cryopreservation, it may be beneficial to freeze sperm in the presence of a cryoprotectant and TEST-yolk buffer supplemented with additional lipids. These lipids could include cholesterol to adjust the cholesterol:PL molar ratio to one, SGG to minimize the entry of water into the sperm membrane during freezing and increase fertilizing ability post-thaw, and plasmalogens and other antioxidants to protect PL-bound PUFAs from oxidation.

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APPENDIX

A.1 Flow chart of amount of lipids required in lipid assays

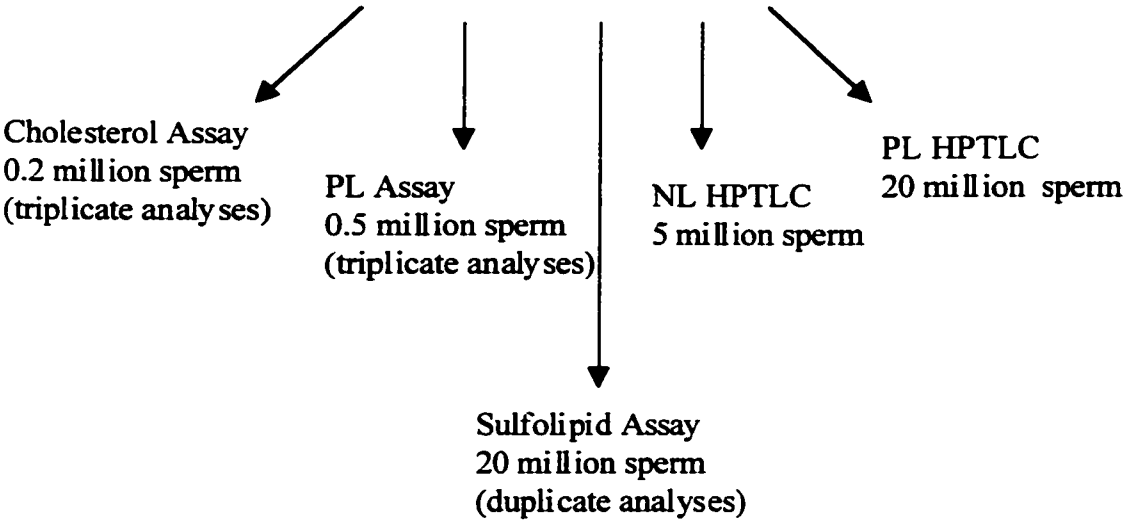
Figure A.1: Flow chart of the amount of lipids required in the various lipid assays in washed non-capacitated and washed capacitated sperm (a) and PGC capacitated sperm (b).

APPENDICES

A.1 Amount of Sperm Required for Each assay

a) Washed and Washed Cap Sperm

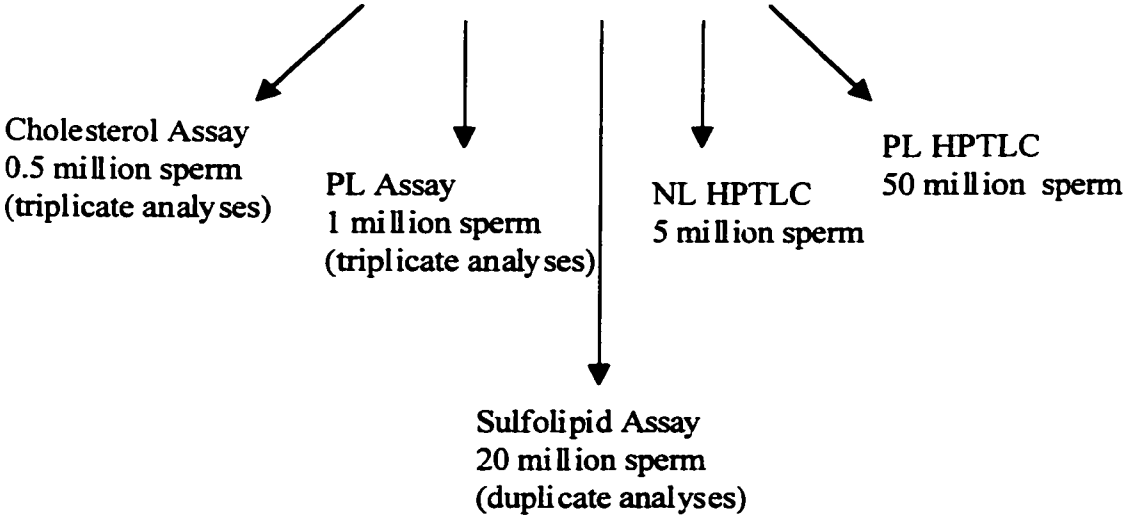
LIPID ANALYSIS: Approximately 75 million sperm (about 3 mice)



FAME ANALYSIS: -a separate batch of lipids was prepared
- approximately 200 million sperm per analysis (about 5 to 8 mice)

b) PGC Cap Sperm

LIPID ANALYSIS: Approximately 100 million sperm (about 25 mice)



FAME ANALYSIS: -a separate batch of lipids was prepared
- approximately 400 million sperm per analysis (about 70 to 80 mice)

A.2 Flow Charts of Assays (as described in Chapter 2)

Figure A.2: Flow chart of the DNA assay. This assay was based on the binding of Hoechst 33258 dye to DNA of live sperm cells that were pre-treated with DTT to decondense the highly condensed sperm chromatin.

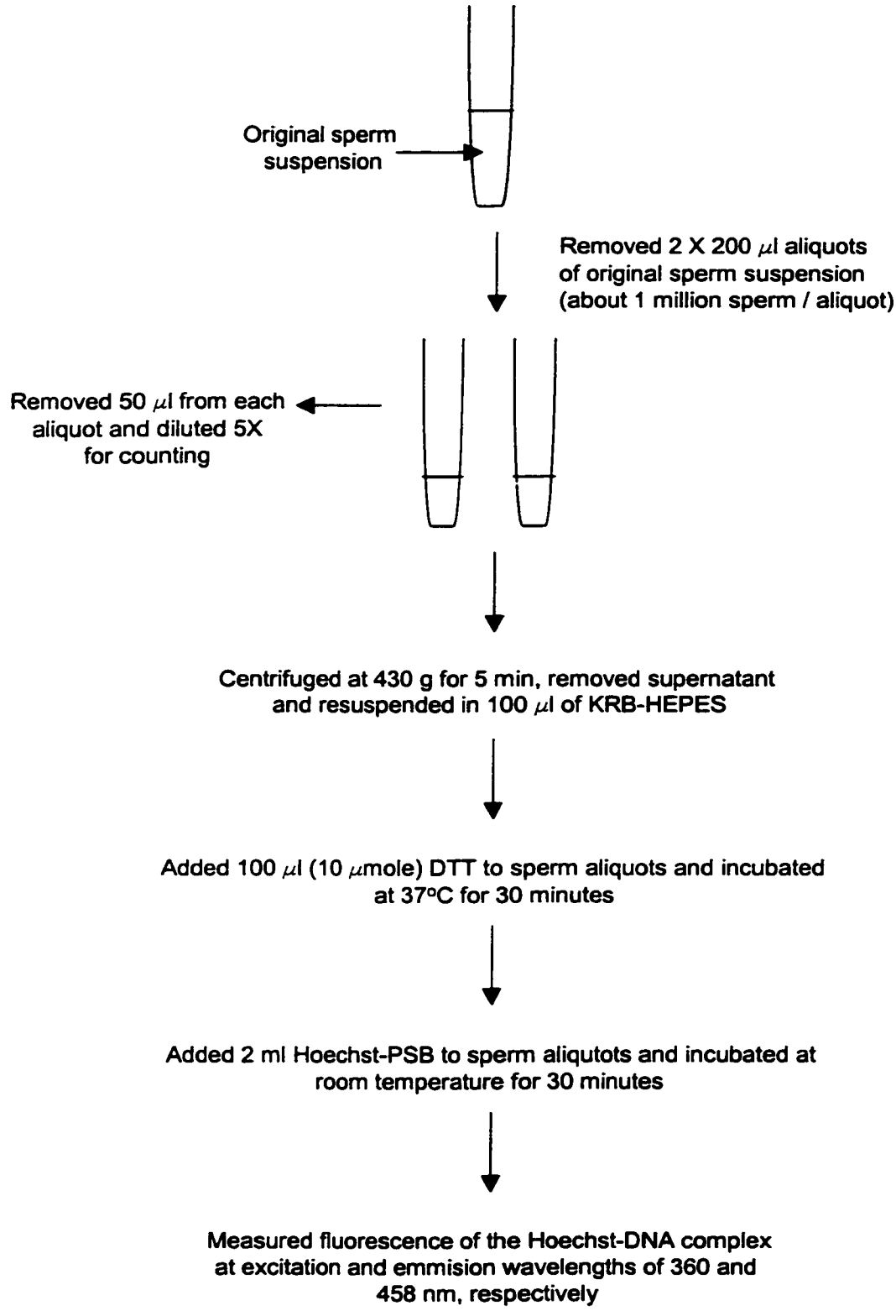
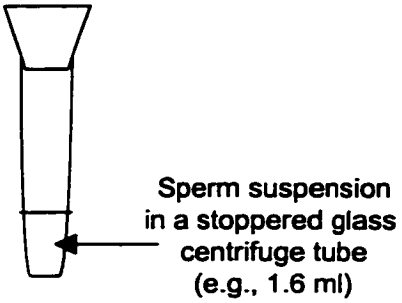
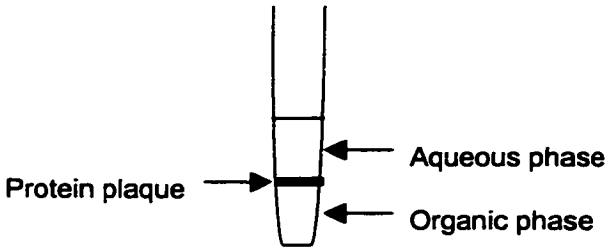

Original sperm suspension
Removed 2 X 200 μl aliquots of original sperm suspension (about 1 million sperm / aliquot)
Removed 50 μl from each aliquot and diluted 5X for counting
Centrifuged at 430 g for 5 min, removed supernatant and resuspended in 100 μl of KRB-HEPES
Added 100 μl (10 μmole) DTT to sperm aliquots and incubated at 37°C for 30 minutes
Added 2 ml Hoechst-PSB to sperm aliqutots and incubated at room temperature for 30 minutes
Measured fluorescence of the Hoechst-DNA complex at excitation and emmision wavelengths of 360 and 458 nm, respectively

Figure A.3: Schematic of lipid extraction according to the method of Bligh and Dyer (1959).

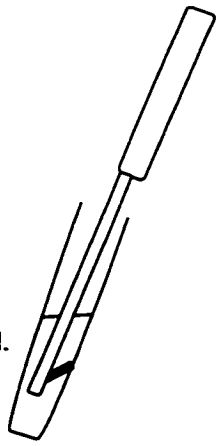


1:2 chloroform:methanol was added to give a ratio of
1:2:0.8 chloroform:methanol:water
(e.g., 2 ml:4 ml:1.6 ml of $CHCl_3$:MeOH:H_2O)

Equivalent volumes of chloroform and water were added to give a two phase system and a final ratio of 1:1:0.9 chloroform:methanol:water
(upper aqueous phase, lower organic phase)
(e.g. added 2 ml each of $CHCl_3$ and H_2O to give
4 ml:4 ml:3.6 ml $CHCl_3$:MeOH:H_2O)



The tube was tipped at an angle so that the protein plaque wasn't touching the side of the tube. The lower organic phase was carefully removed using a pasteur pipet and a volume of chloroform equal to the original volume of chloroform was added.



The lower phases were pooled together and concentrated under N_2 at 40°C. The lipids were stored at –20°C in a teflon-lined screw-capped or a stoppered glass tube.

Figure A.4: Flow chart of the PL assay. PL was quantitated according to the method of Duck-Chong (1979) as previously modified (Tanphaichitr et al., 1996) and was based on PL digestion with $Mg(NO_3)_2$ to inorganic phosphate at high temperature followed by a color reaction with molybdate and malachite green.

An aliquot of total lipid, extracted from about 0.5 million sperm, was added to an assay tube containing 10 μl of 10% $Mg(NO_3)_2$ and concentrated under N_2.

The dried sample was ashed in the blue portion of a bunsen flame

Heating in the bunsen flame resulted in ashing of the organic material, leaving inorganic phosphate.

Added 300 μl of 1M HCl. Heated assay tubes at 90°C for 15 minutes (covered tubes with marbles).

The tubes were allowed to cool and 10 μl of 1% Triton X-100 and 600 μl molybdate/malachite green dye reagent was added.

The absorbance of the greenish blue colour complex was measured at 650 nm.

Figure A.5: Flow chart of the cholesterol assay. Cholesterol was assayed employing to the fluorometric method of Gamble et al. (1978), as previously described (Tanphaichitr et al., 1996), using cholesterol oxidase, peroxidase, and the substrate *p*-hydroxyphenylacetic acid.

Aliquoted total lipid in chloroform,
extracted from about 2 million sperm, to
an assay tube and dried under N_2.

Resuspended lipids in methanol (about 40 μl) and aliquoted
10 μl, consisting of about 0.2 to 0.5 million sperm,
in triplicate, into the wells of a 96 well black plate

Added 100 μl of reagent solution and heated at 37°C
for 30 minutes and then left at room temperature for 15 minutes

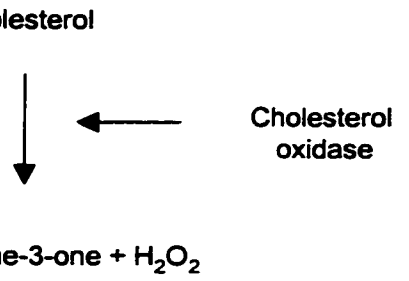
The fluorescence of the stable product was
measured at excitation and emission wavelengths
of 325 nm and 415 nm, respectively.

The reagent solution consisted of:

- 4 ml 0.1 M potassium phosphate buffer (pH 7.4)
- 1 ml cholesterol oxidase (1 U/ml)
- 1 ml peroxidase (10 U/ml)
- 0.5 ml 0.5% Triton X-100 in potassium phosphate buffer
- 0.5 ml sodium cholate (8.6 mg/ml in potassium phosphate buffer)
- 1.5 ml *p*-OH phenylacetic acid (6 mg/ml in potassium phosphate buffer)

Figure A.6: Schematic of the chemical reactions resulting in the formation of the stable fluorescent product in the cholesterol assay.

REACTION 1:



REACTION 2:

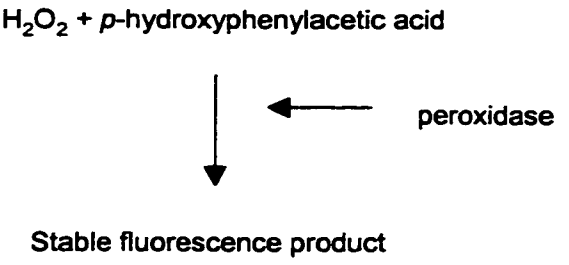


Figure A.7: Flow chart of the sulfolipid assay for SGG quantification, according to the method of Kean (1968) and was based on the formation of a colored complex between the cationic dye Azure A to sulfolipids that is extractable in chloroform.

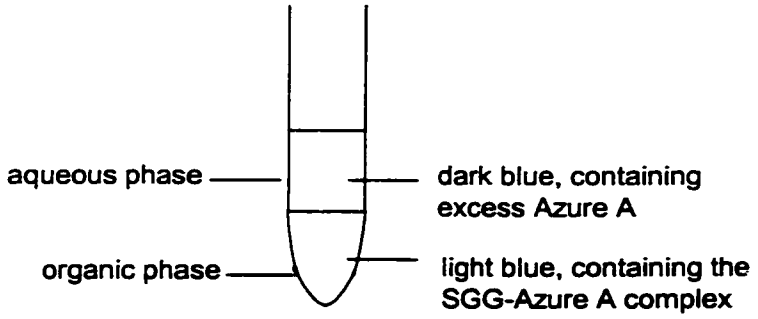
Aliquoted total lipids (equivalent to about 20 million sperm) into 15 ml teflon-lined screw-capped test tubes

↓ ← Dried under N_2

Added 1:1 chloroform:methanol, 0.05 M H_2SO_4 and Azure A Dye solution

↓ ← Vortexed each tube for 30 seconds

Transferred lower phase with a pasteur pipet to a 0.5 ml quartz cuvette and measured absorbance at 635 nm



A.2 Raw Data of GC Results

Table A.1: Raw data for the molar distribution of the major PC fatty acyl chains of washed mouse sperm

FAME	Sample 1[1]	Sample 2[1]	Sample 3[1]	Average[2]
16:0	16.70	13.47	15.63	15.27 ± 1.56
18:0	30.80	31.63	30.33	30.92 ± 0.74
18:1n9	8.03	8.58	9.55	8.72 ± 0.79
20:4n6	31.52	27.38	25.71	28.20 ± 2.81
22:5n6	6.28	10.05	9.67	8.67 ± 2.10
22:6n3	6.37	8.56	8.87	7.93 ± 1.39

[1] Sample 1, sample 2 and sample 3 represent three different sample analyses.
[2] Mean ± standard deviation

Table A.2: Raw data of the molar distribution of the major PE fatty acyl chains of washed mouse sperm

FAME	Sample 1[1]	Sample 2[1]	Sample 3[1]	Average[2]
16:0	15.95	19.72	20.90	18.86 ± 2.53
18:0	16.84	19.30	27.81	21.32 ± 5.45
18:1n9	15.34	14.73	11.36	13.81 ± 2.21
20:4n6	36.24	32.79	31.33	33.54 ± 2.26
22:5n6	6.98	6.30	3.45	5.58 ± 1.83
22:6n3	6.19	6.27	3.99	5.48 ± 1.26

[1] Sample 1, sample 2 and sample 3 represent three different sample analyses.
[2] Mean ± standard deviation

Table A.3: Raw data of the molar distribution of the major SPH fatty acyl chains of washed mouse sperm

FAME	Sample 1[1]	Sample 2[1]	Sample 3[1]	Average[2]
16:0	29.75	42.62	45.64	39.33 ± 8.44
18:0	11.93	17.55	23.76	17.75 ± 5.92
18:1n9	23.17	7.22	7.07	12.49 ± 9.25
20:4n6	3.24	2.30	7.17	4.24 ± 2.58
24:0	14.78	14.03	6.60	11.80 ± 4.52
24:1	12.70	12.51	4.68	9.96 ± 4.58

[1] Sample 1, sample 2 and sample 3 are three separate sample analyses.
[2] Mean ± standared deviation.

Table A.4: Raw data of the molar distribution of the major PC fatty acyl chains of washed capacitated mouse sperm

FAME	Sample 1		Ave.[2]	Sample 2		Ave.[2]	Average[3]
	a[1]	b[1]		a[1]	b[1]		
16:0	18.30	24.07	21.18	20.64	19.32	19.98	20.58
18:0	27.49	24.21	26.42	31.13	31.59	31.36	28.89
18:1n9	8.49	8.48	8.48	8.07	8.43	8.25	8.36
20:4n6	24.45	20.21	22.33	20.78	20.91	20.84	21.58
22:5n6	11.93	9.88	10.90	10.74	11.15	10.94	10.92
22:6n3	9.61	7.45	8.53	8.57	8.53	8.55	8.54

[1] a and b represent duplicate analyses of the same sample preparation.
[2] Represents the average of duplicate analyses, i.e., a and b.
[3] Represents the average of the average of duplicate analyses for each sample.

Table A.5: Raw data of the molar distribution of the major PE fatty acyl chains of washed capacitated mouse sperm

FAME	Sample 1			Sample 2			Average[3]	Sample 3[4]
	a[1]	b[1]	Ave.[2]	a[1]	b[1]	Ave.[2]		
16:0	40.02	33.77	36.90	50.04	42.54	47.28	42.09	50.83
18:0	54.63	50.22	52.42	47.81	58.10	53.9	53.16	45.02
18:1n9	2.51	4.63	3.57	0.70	0.08	0.38	1.98	4.02
20:4n6		8.71						0.13
22:5n6		1.52						
22:6n3	2.69	1.16	1.92	0.14	0.11	0.12	1.02	

[1] a and b represent duplicate analyses of the same sample preparation.
[2] Represents the average of duplicate analyses as analyzed by GC at the University of Guelph.
[3] Represents the average of the average of duplicate analyses for each sample as analyzed by GC at the University of Guelph.
[4] Represent the molar distribution as analyzed by GC-MS at Health Canada.

Table A.6: Raw data of the molar distribution of the major SPH fatty acyl chains of washed capacitated mouse sperm

FAME	Sample 1		Ave.[2]	Sample 2		Ave.[2]	Average[3]
	a[1]	b[1]		a[1]	b[1]		
14:0	6.20	3.02	4.61	5.97	5.01	5.49	5.05
16:0	66.03	56.02	61.03	49.02	55.68	52.35	56.69
18:0	10.20	10.52	10.36	19.35	11.22	15.29	12.83
18:1n9	7.45	1.06	4.26	15.73	5.57	10.65	7.46
20:4n6	2.07	1.89	1.98	0.27	1.35	0.81	1.40
24:0	3.44	13.07	8.26	6.69	11.69	9.19	8.73
24:1	4.09	12.42	8.26	1.67	6.77	4.22	6.24

[1] a and b represent duplicate analyses of the same sample preparation.
[‡] Represents the average of duplicate analyses.
[+] Represents the average of the average of duplicate analyses for each sample.

Table A.7: Raw data of the molar distribution of the major PC fatty acyl chains of PGC capacitated mouse sperm

FAME	Sample 1[1]	Sample 2[1]	Average
16:0	17.06	25.21	21.14
18:0	22.21	24.06	23.14
18:1n9	14.98	11.95	13.46
20:4n6	2.21	4.92	3.56
22:5n6	23.55	19.06	21.30
22:6n3	19.48	13.99	16.74

[1] Sample 1 and sample 2 are separate samples analyses.

Table A.8: Raw data of the molar distribution of the major PE fatty acyl chains of PGC capacitated mouse sperm

FAME	Sample 1[1]	Sample 2[1]	Average
16:0	39.13	42.55	40.84
18:0	18.81	19.24	19.02
18:1n9	24.56	23.53	24.04
20:4n6	8.59	6.86	7.72
22:5n6	5.40	3.95	4.68
22:6n3	3.39	3.69	3.54

[1] Sample 1 and sample 2 are separate samples analyses.

Table A.9: Raw data of the molar distribution of the major SPH fatty acyl chains of PGC capacitated mouse sperm

FAME	Sample 1[1]	Sample 2[1]	Average
14:0	5.22	4.06	4.64
16:0	24.41	55.15	39.78
18:0	6.67	28.78	17.73
18:1n9	61.88	10.11	36.00
20:4n6	0.36	1.24	0.80
24:0	0.17	0	0
24:1	0	0	0

[1] Sample 1, sample 2 and sample 3 are separate sample analyses.

A.4 Basic Theory of Chromatography

"Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction" (IUPAC, 1993). In chromatography, components having similar chemical and physical properties are placed in a system of two physically distinct phases - a mobile phase (i.e. solvent) and a stationary phase (i.e. matrix or support). The components separate because they differ in their distribution between these two phases. The relative movement of each molecule is the result of a balance between the driving force of the mobile phase and the retarding effects of the stationary phase (Freifelder 1982). A dynamic equilibrium occurs between the phases that can be characterized by the distribution ratio (K), and is defined as the ratio of the amount or concentration of a given component in the stationary phase (C_s) to that in the mobile phase (C_m) per unit volume:

$$K = C_s / C_m,$$

(Braithwaite and Smith, 1996). Each component in a mixture has a different value for K, reflecting its relative affinity for the stationary phase. A higher value of K indicates a greater affinity for the stationary phase.

A component is considered to move through the column or plate in a series of theoretical steps, with the distribution between the two phases being defined by K. The resolution between components is a function of the number of separation, or equilibrium, steps in a column or plate, and the dispersion of molecules during the separation process. The more steps, e.g. the longer the column or plate, the better the resolution. Separation of the components in a mixture occurs because the components exhibit different relative

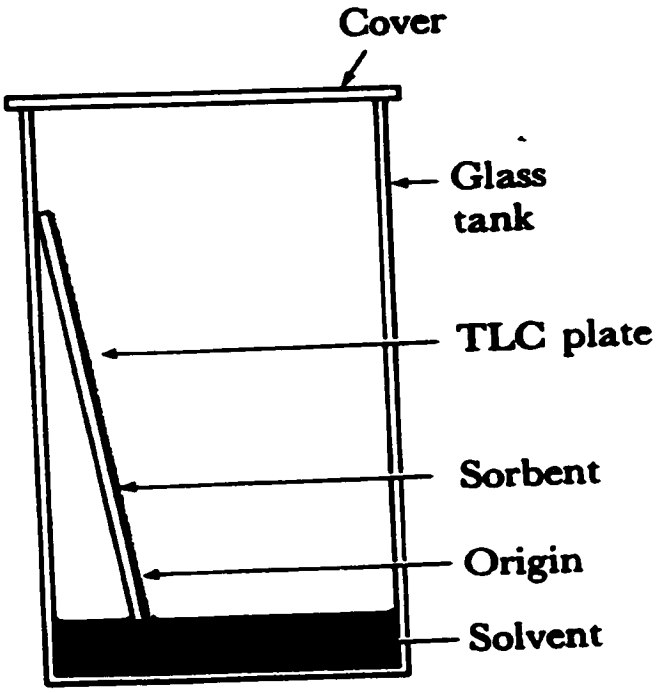
distributions and migrate at different rates. Factors influencing the retention of a component through the column or plate include the composition and properties of the mobile phase, the type and properties of the stationary phase, the intermolecular forces between the components and the stationary and mobile phases, and in some cases, the temperature. If resolution is insufficient, varying selectivity towards either the stationary or the mobile phase by changing elution conditions may be required.

A.3.1 Thin Layer Chromatography (TLC)

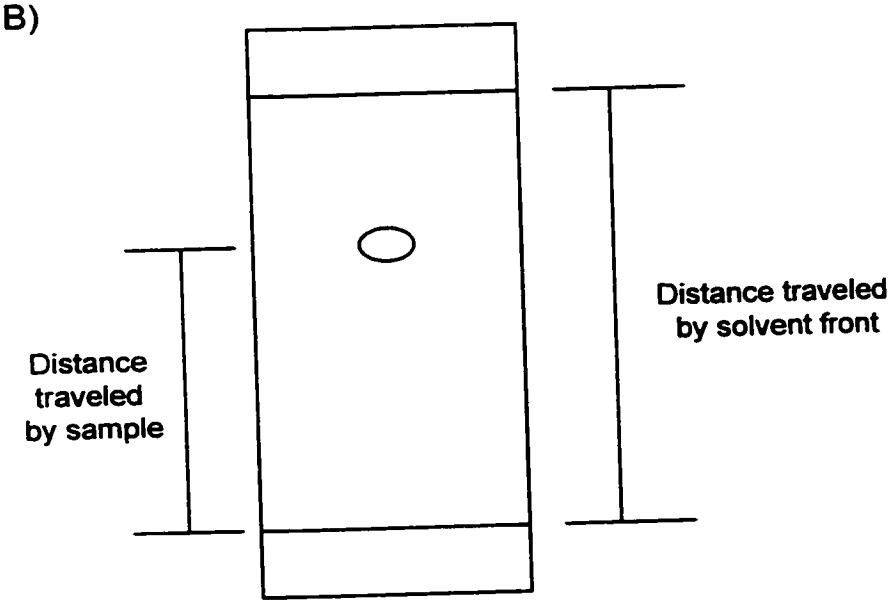
Separation by TLC involves distribution of sample components between the adsorbent (stationary phase), which is held on plastic or glass plates in a thin layer (0.25 to 0.5 mm), and the developing solvent (mobile phase). Important properties of the adsorbent include the particle size and homogeneity, and the most commonly used adsorbent is a very fine grade silica gel (particle size of 5 to 17 μm) (Braithwaite and Smith, 1996). TLC can be used to separate a variety of samples, including lipids, amino acids and sugars (Braithwaite and Smith, 1996). Samples are applied as a spot or band about 1.5 to 2 cm from the bottom of the plate using a syringe or sample applicator, and the plate is placed in a tank containing the mobile phase solvent such that the applied sample is above the solvent level. The plate is developed by ascending chromatography (Figure A.1A), i.e., the solvent moves up the plate by capillary action, taking the various components with it at differing rates depending on the extent to which they adsorb to the stationary phase versus their solubility in the mobile phase, i.e., according to their distribution ratios (Braithwaite and Smith, 1996). When the solvent nears the top of the plate, the plate is removed and dried in air or under nitrogen to remove the solvent.

Figure A.8: A) TLC plate development by ascending chromatography. Adapted from Freifelder, 1982. B) Determination of R_f.

A)



B)



$$R_f = \frac{\text{distance traveled by sample}}{\text{distance traveled by solvent front}}$$

The TLC plate is placed in a jar with a tight-fitting lid to avoid evaporation of the developing solvent. Filter paper is used to line the sides of the jar to maintain an environment that is saturated with solvent. Developing solvent is added to the jar in the appropriate ratio so that it is 0.5 to 1 cm from the bottom of the jar and the jar is left for about 30 minutes to let the atmosphere get saturated with solvent. A variety of solvent systems can be used depending on the sample to be separated. For example, the major PL classes can be separated using a solvent system of $CHCl_3$:MeOH:H_2O (65:25:4, v/v/v) (Kates, 1986) or $CHCl_3$:MeOH:acetic acid:H_2O (50:37.5:3.5:2, v/v/v/v) (Holub and Watson, 1996). The major neutral lipids can be separated using a solvent system of petroleum ether:ethyl ether:acetic acid (80:20:1, v/v/v) (Kates, 1986).

Following plate development, a variety of methods can be used to detect the separated lipid components. The quickest method of visualization is to place the plate in a tank of iodine for a few minutes, which causes all lipids to appear as brown spots that fade over time. A variety of lipid sprays can also be used. For example, spraying the plate with 50% sulfuric acid and heating at 100°C for 5 to 10 minutes makes lipids visible as black deposits of carbon (Christie, 1982). A 0.1% solution of 2', 7'-dichlorofluorescein in 95% methanol is a non-specific reagent and causes lipids to appear as yellow spots under UV light. A variety of sprays may also be used to detect specific lipids, such as orcinol, which detects the carbohydrate moieties of glycolipids, ninhydrin, which detects amine groups, and azure A, which detects sulfated lipids (Christie, 1982). The identity of components can be compared to standards by determining their migration relative to the solvent front (R_f) (Braithwaite and Smith, 1996), which is related to the distribution coefficient of the components. The R_f is calculated by taking the ratio of the

distance traveled by the component and the distance traveled by the solvent front (Figure A.1B).

Advantages of TLC include high speed of separation (most separations can be completed in less than 1.5 hours), easy detection of sample components and easy isolation of components from the plate. Samples can be recovered from TLC plates by scraping the silica containing the sample of interest off the plate into a test tube, and eluting the sample from the silica using the appropriate solvent.

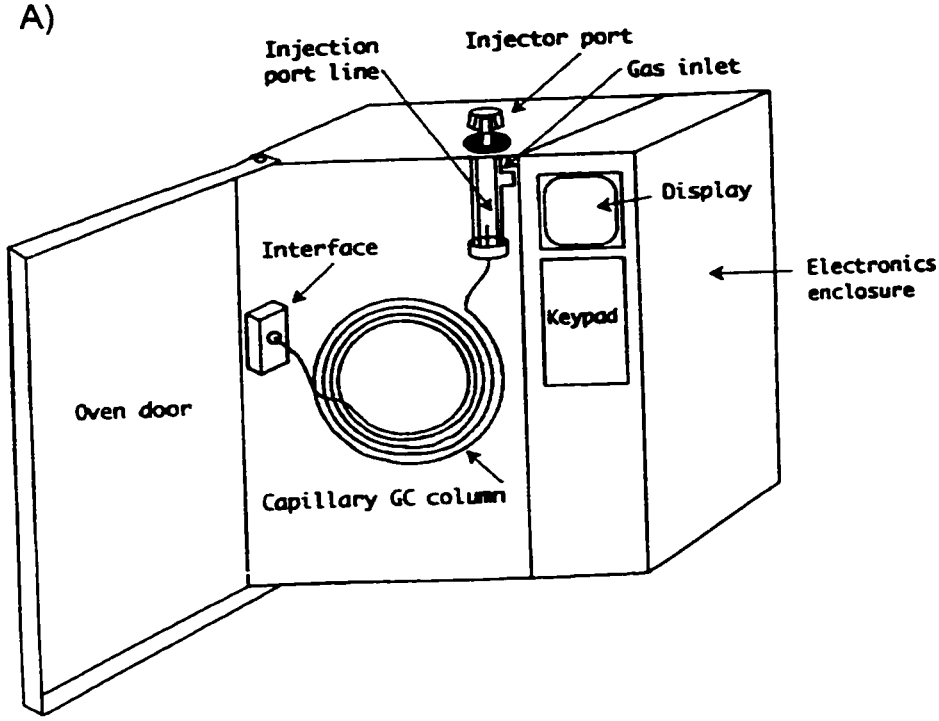
High perfomance thin layer chromatography (HPTLC) has been employed to give separation efficiencies about three times higher than normal TLC. The adsorbent consists of silica gel of smaller and more uniform particle size (2 to 10 μm) and a thinner layer thickness (100 μm) than is generally employed. With these plates, both the resolution and speed of separations are improved and smaller quantities of sample are required.

A.4.2 Gas Chromatography

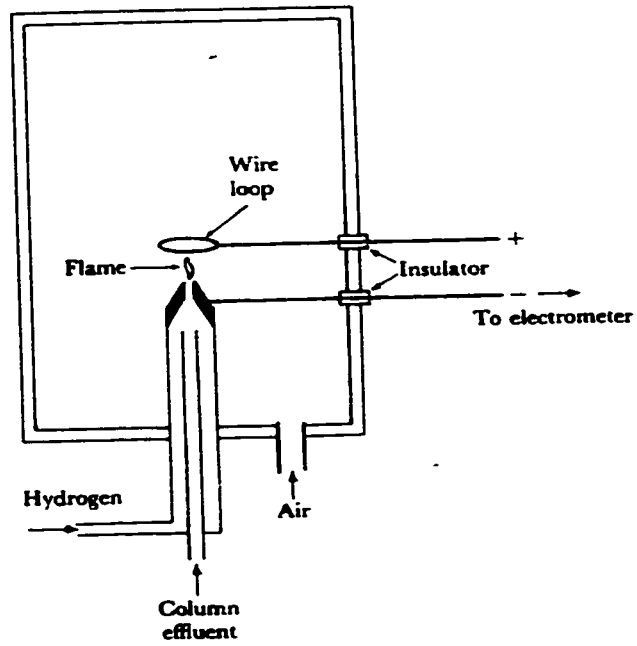
Separation by gas chromatography (GC) occurs as a result of absorptive interactions between the components in a gas stream and the column coating (McMaster and McMaster, 1998). The compounds to be separated are volatilized at high temperature and passed in a stream of inert gas (mobile phase, usually helium, nitrogen or argon) through a column in which a high boiling point liquid is coated onto a solid supporting material (stationary phase) (Christie, 1982). The GC consists of a carrier gas source and control valve, a temperature control oven, and an injector that is connected to the column (Figure A.2A). The column is a 25 to 150 meter coiled capillary tube with an internal diameter of 0.25 to 0.75 mm (McMaster and McMaster, 1998). A variety of

Figure A.9: A) Components of a GC. Adapted from McMaster and McMaster, 1998. B) The flame ionization detector. Adapted from Freifelder, 1982.

A)


Injection port line
Injector port
Gas inlet
Display
Interface
Electronics enclosure
Keypad
Oven door
Capillary GC column

B)


Wire loop
+
Flame
Insulator
To electrometer
Hydrogen
Air
Column effluent

stationary phases having different chemical compositions are available, but the most common film or bonded phase is a non-polar material, such as methyl silicone. This packing is stable, has a high capacity and provides a separation that parallels the compounds boiling point, i.e., low boilers come off first, while high boilers come off last (McMaster and McMaster, 1998). Other column packings include Carbowax, which separates compounds according to carbon number, i.e., the more carbons, the longer it is retained (McMaster and McMaster, 1998). Only compounds that are capable of being volatilized without decomposition and having a molecular weight up to 400 to 500 can be analyzed by GC (Christie, 1982), and the separation is based on the distribution ratios of the sample components, which are dependent on their volatilities and on their relative solubilities in the liquid phase (Christie, 1982). The sample is introduced as a liquid with an inert gas and the gaseous mixture is passed through the column tubing. As fresh carrier gas is flushed down the column, the vaporized components are continually redistributed between the gaseous mobile phase and the liquid stationary phase according to their distribution ratios. The substances emerge from the column as peaks of concentration that are detected and converted in the gas phase to an electrical signal. The signal is amplified and passed on to a continuous recorder to obtain a tracing.

After introducing the sample through the injector, there is a small air peak followed by a solvent peak, after which the baseline soon stabilizes and the sample components begin to emerge. The time from the point of injection to that when the maximum amount of each component has emerged, is the retention time of the substance. Various column parameters such as gas flow rate, length of column and composition of the stationary phase can be chosen to optimize the maximum resolution of peaks

(Braithwaite and Smith, 1996).

The flame ionization detector is the most frequently used mode of detection (Figure A.2B). The eluted components are burned in a flame of hydrogen gas and air. Any carbon in the sample is burned and converted to carbon dioxide (Freifelder, 1982). For unclear reasons, electrons and negative ions are produced and detected as a current, which is then converted into voltage difference. The flame ionization detector is very sensitive and has a good signal-to-noise ratio. Provided the gas flow rates are constant during the analysis, the response of the detector to a homologous series of compounds, e.g. fatty acid methyl esters, is linearly dependent on the mass of each component over a wide range of molecular weights.

Analyte preparation will generally provide an ester or other functional group at the carboxylic portion of the molecule. For example, to determine the fatty acyl chain composition of PLs, the PLs are subjected to acid or base methanolysis to generate fatty acid methyl esters (FAMEs). Quantitative analysis of the individual peaks can be done with the use of an internal standard (Kates, 1986). A known amount of an unusual FAME standard that is not normally found in tissues (e.g. 17:0 or 19:0) is added to the sample FAMEs and the mixture is analyzed by GC. The sample peaks can then be compared back to the known amount of internal standard and the amount of each peak can be calculated.

GC provides excellent separation of small molecules because of the length of the column. It has a high sensitivity and the time for analysis is short. Use of a non-destructive detector or addition of a flow-splitter allows for collection of the peaks, which can be used for further characterization. Analysis by GC indicates the proportion of a

component emerging from the column as a function of time, however, there is no direct indication of the identity of the component producing a particular peak, nor the amount in the peak. In the absence of authentic fatty acid standards, provisional identifications can be made by drawing a straight-line relationship between the logarithm of the absolute retention time and the chain length. With this method, the retention time for a particular fatty acid can be predicted.

CURRICULUM VITAE

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EDUCATION

University of Ottawa / Loeb Health Research Institute
M.Sc. candidate in Biochemistry, expected Winter 2001
Received the Ontario Graduate Scholarship (Sept. 1999 to Aug. 2000)
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University of Ottawa
B.Sc. in Biochemistry, Honors Nutrition, September 1994 to May 1998
Graduated Magna Cum Laude
Deans List for Academic Achievement (1996-1997 and 1997-1998)

SCIENTIFIC WORK EXPERIENCE

Tularik Pharmaceutical Company
Research Assistant, April 2001 to present
Involved in characterizing the roles of various targets in obesity and diabetes using molecular and cellular biology techniques.

University of Ottawa / Loeb Health Research Institute
Graduate student (M.Sc.), September 1998 to present, expected Winter 2001
The major objective of my work involved characterizing the lipid components of mouse sperm before and after capacitation. Techniques used include various chromatographies (TLC, GC, and column chromatography). I have gained experience in working with animals, sperm cell preparation, isolation and handling of lipids, and development and optimization of various assays.

University of Ottawa, Department of Biochemistry
Lab Demonstrator, January 2000 to April 2000
Duties included supervising second year undergraduate students during laboratory experiments and teaching laboratory techniques involved in DNA extraction and characterization. I was also responsible for evaluating students' performances in the laboratory and correcting laboratory reports.

University of Ottawa / Loeb Health Research Institute
Honours student (B.Sc.), September 1997 to April 1988
The major objective was to develop an HPLC method to separate the major phospholipid classes of ram testes and sperm lipids. Techniques used included TLC, and HPLC.

University of Ottawa, Student Services
Tutor, September 1997 to April 1998
Tutored students in various science courses including organic chemistry and biochemistry.

LABORATORY SKILLS

- Handling of mice
- Sperm cell preparation
- Lipid extraction and characterization
- Liquid, thin layer, column and gas chromatographies
- Infrared spectroscopy and liposome preparation
- Lab management

SCIENTIFIC ACTIVITY

Presentations

- Ottawa Reproductive Workshop, Ottawa Hospital (1998 – poster, 1999 and 2000 - oral)
- Annual Conference for the Study of the Society of Reproduction, Madison, Wisconsin (2000 – oral)

Manuscript

- Furimsky, A.M., M. Kates, B. Holub, P. Kumarathasan, R. Vincent and N. Tanphaichitr. Characterization of lipids from washed and Percoll gradient centrifuged mouse spermatozoa. In preparation.

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CONTRIBUTION OF COLLABORATORS

Binding assays were performed with the help of Wattana Weerachatyanukul, and some sperm samples were prepared with the help of Dr. Julierut Tantibhedhyangkul.

HPTLC of the major PL classes of washed, washed capacitated and PGC capacitated sperm lipids was performed with the help of Ali Shoushtarian.

GC analysis of FAMEs was performed in collaboration with Dr. Bruce Holub at the University of Guelph by Jerry Piekarski. Preparation of sperm samples and lipid extraction were performed in Ottawa by Anna Furimsky, and the lipid samples were sent to Guelph for subsequent GC analysis.

MS-MS analysis of 22:5n6 FAME was performed in collaboration with Dr. Tom Brenna at Cornell University by Anthony Michaud.

GC-MS analysis was performed at Health Canada (Ottawa, Ontario) in collaboration with Dr. Renaud Vincent, by Dr. Premkumari Kumarathasan. Sperm sample preparation, lipid extraction, and FAME/DMA and DMA standard generation were performed by Anna Furimsky.