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MODULATION OF THE FLUIDITY AND FUNCTION OF BOAR SPERMATOZOA MEMBRANES

BY

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ABSTRACT

The fertility rates obtained using cryopreserved boar semen are considerably less than those with fresh boar semen. The hypothesis of this project was that commercially used freezing and thawing processes damage the ultrastructure of head plasma membranes (HPM) from boar spermatozoa, thereby reducing the fertilizing capacity of the cell. The aim of this study was to examine the sites and mechanisms of cold-induced spermatozoa injury through the use of various membrane modulators.

Assessment of HPM fluidity over time with the fluorescent probes trans- and cis-parinaric acid (t-PnA and c-PnA), revealed the presence of small domains of differing fluidities, indicaticating the presence of specialized regions within the predominantly fluid-phase bilayer. These domains responded differently to Ca^{2+} at 25C.

When spermatozoa were cooled and extended prior to freezing, t-PnA detected a slight decrease in HPM fluidity and the frozen-thawed HPM underwent a smaller decrease in fluidity. The HPM from neither the cooled nor the frozen-thawed spermatozoa responded to the addition of Ca^{2+}. Fluidities of the sperm body membranes (SBM) from cooled and

frozen-thawed spermatozoa did not differ in the absence of Ca^{2+}. In the presence of Ca^{2+}, however, the cooled SBM displayed an increase in fluidity which was less pronounced than that of the frozen-thawed SBM.

The HPM isolated from cold shocked spermatozoa or the HPM from normal spermatozoa exposed to phospholipase A_2 from either bee or snake venom fluidized over time at 30C. Inclusion of defatted bovine serum albumin with the HPM containing phospholipase A_2 from bee venom or snake venom resulted in an initial decrease in overall HPM fluidity, although the HPM underwent a time-related fluidization.

Intact, cold shocked spermatozoa were unable to regulate intracellular Ca^{2+}, as measured by a $^{45}Ca^{2+}$ radioassay. Bee venom phospholipase A_2 increased Ca^{2+} transport from the cell and snake venom phospholipase A_2 did not affect spermatozoa Ca^{2+} flux.

Cold-induced boar spermatozoa damage may be related to, but is not the same as the cellular injuries inflicted by phospholipase A_2 from bee or snake venom. These findings, however, lead to some suggestions regarding the locations and mechanisms of damage to boar spermatozoa membranes caused by low temperature.

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LITERATURE REVIEW

1.1 INTRODUCTION

Artificial insemination (AI) is practised world-wide on a number of domestic species (Bonadonna and Succi, 1976; Foote, 1982; Reed, 1985). The popularity of AI is attributed to the simplicity of the techniques involved, and to the advantages it provides to the producer (Reed, 1982; Bearden and Fuquay, 1984), including:

1. availability of progeny-tested genetically superior sires to all producers at a reasonable cost;
2. maintenance of high health status of the herd or flock;
3. reduced contact with expensive and potentially dangerous males;
4. alleviation of breeding difficulties between the male and female;
5. maximized exchange of genetic material.

These advantages are expanded with long-term semen

preservation. In 1776, Spallanzini observed that when human, stallion, and frog spermatozoa were cooled in snow for 30 minutes, they became inactive but were revived on warming (Watson, 1979). The first suggestion of frozen semen banking was made by Mantegazza in 1886 (Sherman, 1978) who noted the benefits of freezing horse and cattle sperm for transportation. Mantegazza's predictions began to be fulfilled in 1949 when Polge et al. reported that bovine spermatozoa were capable of withstanding freezing in the presence of glycerol. Since then, cryopreservation of bovine spermatozoa has been improved, but the application of these techniques to spermatozoa of other species has produced variable and often disappointing results (Berndtson and Pickett, 1978; Polge, 1980). In particular, the response of boar spermatozoa to freezing and thawing is very poor (Paquignon, 1983; Reed, 1985). The fertility and prolificacy levels following insemination with cryopreserved boar semen are considerably lower than those with extended fresh boar semen (Corteel and Paquignon, 1984). Insemination with extended fresh semen gives farrowing rates of 91.7% with 10 piglets per litter using one dose of 3 billion total fresh spermatozoa (Maynard et al., 1987). Insemination with frozen semen gives a 54.2% farrowing rate, 8 piglets per litter using 6 billion total spermatozoa (Maynard et al., 1987). Frozen-thawed semen is therefore unattractive to most producers.

1.2 METHODS OF FREEZING BOAR SEMEN AND THE RESULTS

1.2.1 Characteristics of Semen Diluents

For AI, semen is extended in a diluent prior to its use. An ideal diluent should have several important qualities (Graham, 1978), including:

1. appropriate osmolality and electrolytic balance to prevent any deleterious ionic effects of the spermatozoa;
2. metal-complexing properties that can bind heavy metals harmful to spermatozoa;
3. a pK around 7, providing a buffering ability to prevent shifts in pH during cooling and formation of lactic acid (a biproduct of sperm metabolism);
4. stability to resist enzymatic and/or nonenzymatic degradation;
5. the ability to substantially increase the seminal volume so multiple inseminations can be performed;
6. appropriate environment for normal spermatozoa metabolism.

A semen diluent for cryopreservation, in addition to such functions, must contain protective agents against the effects of cooling and freezing (Graham, 1978).

1.2.2 Components of Diluents

Currently, there are different methods used commercially for freezing boar semen (Pursel and Johnson, 1975; Westendorf et al., 1975 in Larsson, 1978; Paquignon and Courot, 1976; Larsson et al., 1977), but the principles among them are similar. The diluents contain energy sources, buffers, adjuvants, and cryoprotectants. Glucose, fructose, and lactose are the main energy sources. Orvus ES Paste (O.E.P.), a surfactant sodium lauryl citrate, is included between 1-2% in the diluents of Pursel and Johnson (1975); Westendorf et al. (1975 in Larsson, 1978), and Larsson et al. (1976). Although its mechanism of action is not fully understood, O.E.P. appears to preserve the integrity of the spermatozoa (Larsson, 1978) and increases post-thaw motility (Graham and Crabo, 1972). All four methods use approximately 20% hen's egg yolk as a cryoprotectant to protect the spermatozoa during the freeze-thaw process (Graham and Crabo, 1972; Paquignon, 1983). The low density lipoprotein fraction of egg yolk appears to be the protective agent (Polge, 1980). The cholesterol in the egg yolk may contribute to plasma membrane stability during the freezing process by altering its fluidity (Watson, 1981). The O.E.P. may alter egg yolk constituents, and enhance the benefits of the egg yolk as a cryoprotectant (Pursel et al., 1978a). Glycerol is present in all diluents at a level of 2% and appears to be the most efficient cryoprotectant for boar spermatozoa.

Early studies indicate that at levels of 4-8%, glycerol maximized post-thaw motility, but not fertilizing capacity, of frozen-thawed boar spermatozoa (Polge, 1956; King and McPherson, 1967; Wilmut and Polge, 1977a, 1977c). Acrosomal damage also increased with glycerol concentration (Wilmut and Polge, 1977a; Murdoch and Jones, 1978). In the presence of 1-2% glycerol, post-thaw motility was reduced, but the spermatozoa retained better fertilizing capacity (Wilmut and Polge, 1977a). Glycerol at 2% was found to improve the spermatozoa recovery rate, but increase the release of glutamic oxalacetic transaminase (GOT) from the cell, perhaps decreasing the spermatozoa life span (Graham and Crabo, 1972). The GOT is an enzyme involved in the activation of acrosomal enzymes, and hence is required for fertilization. Sanford et al. (1972) showed that the oxygen uptake of frozen-thawed boar spermatozoa was lower than that of fresh spermatozoa and that cell respiration decreased as glycerol concentration increased. These authors postulated that cryopreserved boar spermatozoa could not remain motile or viable long enough in the female to ensure normal fertilization rates unless inseminated via the uterine horn or oviduct. It has been suggested that since spermatozoa membranes are highly permeable to glycerol, it may stabilize the membranes to an extent that reduces their ability to capacitate and undergo the acrosome reaction (Murdoch and Jones, 1978). Watson (1981) also suggested that the site of action of glycerol is

at the sperm plasma membrane level.

1.2.3 Processes of Preservation

There are similarities in the freezing processes of the four commercial methods, including:

1. equilibration of spermatozoa in seminal plasma or diluent during cooling;
2. slow cooling to 8-5C over a 4.5 to an 8 hour period;
3. concentration of semen by centrifugation;
4. freezing to -196C at an appropriate spermatozoa concentration to preserve the semen quality (3-6x10^9 spermatozoa per insemination).

The thawing procedures all involve very fast thaw rates in warming solutions of approximately 50C. Such rapid heating may minimize spermatozoal damage during thawing (Pursel and Johnson, 1975; Larsson et al., 1976). Current studies have demonstrated that boar semen cooled to only 5C suffered severe damage following warm shock, when the 5C semen was rapidly diluted with a 37C solution (Bamba and Cran, 1985, 1988). The authors of these individual studies all state that based on fertility trials, each method yields acceptable fertility levels (Pursel and Johnson, 1975; Westendorf et al., 1975 in Larsson, 1978; Paquignon and Courot, 1976; Larsson et al., 1977). In the successful fertility tests, however, the number of motile frozen-thawed spermatozoa per insemination dose has

been considerably greater than required for fresh semen AI (Larsson, 1978; Paquignon, 1983; Reed, 1985; Maynard et al., 1987). In addition, farrowing results from numerous fertility trials indicate that these methods result in approximately 55% farrowing with litter sizes of about eight, which is inadequate for commercial swine production (Johnson, 1985).

1.2.4 Identifying the Problem

Based on the high concentration of frozen-thawed spermatozoa required per insemination, and on the subsequent fertility levels achieved, it is evident that cryopreservation methods currently used damage spermatozoa. Proper identification of cell injury is crucial as it would permit viability tests resulting in the disposal of severely damaged samples and better fertility rates. More importantly, understanding the mechanisms and location of cryo-induced damage will lead to improved preservation techniques.

Freezing and thawing reduce the percentage of motile spermatozoa by at least 50% (Larsson, 1985) and in fertility trials, frozen-thawed ejaculates with less than 15% (Paquignon et al., 1980) or 35% (Johnson et al., 1981) progressively motile spermatozoa have been discarded. Post-thaw motility, however, is not correlated with fertility (Wilmut and Polge, 1971, 1977a, 1977b; Pursel et al., 1972) and post-thaw immotility can be overcome by the addition of caffeine (Larsson et al., 1976). Thus, motility of cryopreserved

spermatozoa is not a valid index of fertility.

The acrosome is crucial to fertilization. Boars having abnormal acrosomal morphologies are often subfertile (Gibson, 1983). Hurtgen et al. (1980) observed that in eosin nigrosin stained smears of fresh semen, the eosinophilic ("dead") cells usually displayed a lifting or full loss of the acrosome. Acrosomal morphology has been widely used as a viability indicator for post-thaw spermatozoa (Pursel et al., 1978a, 1978b; Ibrahim and Kovacs, 1982; Bamba and Cran, 1985). Cryopreservation of spermatozoa caused an increase in acrosomal abnormalities (Larsson et al., 1976; Potter et al., 1979; Ibrahim and Kovacs, 1982). In post-thaw spermatozoa, however, the relationship of percent normal apical ridge (NAR) on the acrosome to fertility is questionable (Larsson et al., 1976; Wilmut and Polge, 1977c; Johnson et al., 1981; Johnson, 1985).

Cold associated injury results in disruption of the selective permeability of the plasma membrane of animal cells (Watson, 1981; Robertson and Watson, 1986; Watson and Morris, 1987). It has recently been suggested that the cell plasma membrane is the primary site for freezing injury (Morris, 1981; Fujikawa and Miura, 1987; McGann et al.,1988). Since the spermatozoa head plasma membrane (HPM) has an integral role in fertilization (Langlais and Roberts, 1985), it is logical to hypothesize that damage to the plasma membrane could alter normal spermatozoa functions that influence

fertility. Freeze-thaw survival of the spermatozoon is, in fact, largely dependant on the response of the plasma membrane (Koehler, 1985). The molecular structure of the plasma membrane is altered during cooling and rewarming (Canvin and Buhr, 1988). Identification and comprehension of freeze-thaw damage to the spermatozoa plasma membrane, therefore, may aid in the understanding of how fertility is lost during frozen preservation.

1.3 FERTILIZATION

The spermatozoa HPM surrounds the outer acrosomal membrane (OAM). The HPM plays a crucial role in fertilization as it is an integral part of the capacitation process and the acrosome reaction (Yanagimachi, 1981; Rogers and Bentwood, 1982; Farooqui, 1983; Langlais and Roberts, 1985). Capacitation is a collection of biochemical events experienced by the spermatozoa during transit in the female tract resulting in the acrosome reaction (Langlais and Roberts, 1985). The acrosome is a cytoplasmic organelle, considered to be a modified lysosome containing a series of enzymes required for fertilization (Shapiro et al., 1981; Yanagimachi, 1981; Rogers and Bentwood, 1982; Peterson and Russell, 1985). It is located directly beneath the HPM and is surrounded by the acrosomal membranes. The acrosome reaction involves the fusion and fenestration of acrosomal contents and exposure of

the inner acrosomal membrane (IAM; Langlais and Roberts, 1985). The freshly ovulated ovum is surrounded by several layers of cells and membranes through which the capacitated spermatozoon must penetrate. The acrosome reaction is triggered near or within the first barrier, the *cumulus oophorous* (Rogers and Bentwood, 1982). Thus, the release of the acrosomal enzymes breaks down the barriers around the ovum, and enables the spermatozoa to reach the ovum (Rogers and Bentwood, 1982).

Capacitation is associated with molecular changes at the HPM level including the alteration of membrane surface proteins, a shift in membrane lipids, and an influx of calcium leading to the acrosome reaction (Langlais and Roberts, 1985). The acrosome reaction involves distinct morphological changes of the spermatozoa head. Initially, there is a swelling of the acrosome which brings the OAM closer to the HPM, facilitating the formation of multiple fusions between the membranes. The fused areas form small vesicles and detach from the spermatozoa head, therefore only the IAM remains and the acrosomal contents are liberated (Yanagimachi, 1981). These alterations of membrane molecular structure and the fusigenic events indicate a dynamic membrane structure which must be capable of undergoing rapid and radical reorganization in order to fulfil the spermatozoon's functions. In order to understand these events preceding fertilization, it is

essential to comprehend the dynamics of the plasma membrane.

1.3.1 Membrane-Function Relationship

1.3.1.1 General Membrane Structure

Current concepts of biological membrane structure are based on the "fluid mosaic" model proposed by Singer and Nicholson (1972). Cell membranes are arranged as a phospholipid bilayer with the hydrophilic polar phosphate moieties at the intra- and extra-cellular surfaces, with their attached hydrophobic fatty acid side chains in the interior of the bilayer. Primary components of cell membranes are phospholipids, sterols and proteins. Peripheral membrane proteins are loosely bound to the outer membrane surface and integral proteins are deeply inserted or span the bilayer. Proteins maintain the structural integrity of the membrane and are also responsible for much of the membrane's biological activity.

In most plasma membranes, the lipids are in the liquid-crystalline (fluid) phase which permits lateral diffusion of membrane components (Pringle and Chapman, 1981; Malhatra, 1983). Without this mobility, the membrane, and hence, the cell, cannot function normally (Shinitzky, 1984).

The fluidity of a bilayer is determined by its lipid composition (Bretscher and Raff, 1975). In the fluid state, the lipids are highly disordered (Lee, 1983) because the fatty

acid chains of the phospholipids are flexible, rotate easily, and the polar ends of some lipids are quite mobile (Chapman, 1983). In the gel state, the phospholipids are arranged in an ordered crystalline lattice with a characteristic hexagonal packing of the extended fatty acid groups (Pringle and Chapman, 1981).

1.3.1.2 Assessing Membrane Fluidity

Molecular probes are used to characterize membrane structure. This is accomplished by a change in some physical property of the probe in response to membrane alterations (Radda, 1971). Sklar et al. (1975, 1977) used polyunsaturated fatty acids with conjugated double bonds as fluorescent probes for membrane systems. The fatty acid nature minimizes the perturbations of the membrane upon introduction of the probe (Sklar et al., 1975). The fluorescent probe responds to the excitation energy released from the molecules surrounding the probe thus measuring the polarity of the probe's binding site. The polarity of membrane molecules reflects the fluidity of the probe's immediate environment. Thus, polarity is a measure of the fluidity of the binding site (Radda, 1971).

Partitioning of a mixed series of phospholipids and the α (cis) and β (trans) isomers of parinaric acid showed that trans-parinaric acid (t-PnA) preferentially associated with gel-phase (solid) lipids, while cis-parinaric acid (c-PnA)

distributed equally between solid and fluid lipids (Sklar et al., 1977; 1980). The fluorescence measurements of membrane-incorporated cis- or trans-parinaric acid revealed phase shifts or transitions that reflected the fatty acid composition of that sample (Tecoma et al., 1977; Sklar et al., 1979; Sklar and Dratz, 1980; Canvin and Buhr, 1988). The coexistence of fluid and solid domains in a membrane has been identified using cis- and trans-parinaric acids (Schroeder, 1983).

1.3.1.3 Factors Affecting Membrane Structure

Any factor which modifies membrane molecular structure or molecular interactions, may influence membrane fluidity. A modulator may act directly on the structure of individual lipids or proteins, or influence interactions among lipids and/or proteins. A number of substances and physical conditions affect membrane structure, including:

1. cholesterol;
2. hydrocarbon chain characteristics;
3. proteins;
4. calcium;
5. temperature.

Cholesterol modulates bilayer structure as its steroid rings interact with the hydrocarbon chains of phospholipids close to the phosphate head group. Thus, head group mobility is restricted while leaving the acyl side chains relatively

flexible. Therefore, cholesterol promotes the close, ordered packing of membrane phospholipids (Darin-Bennett and White, 1977; Koehler, 1985) which rigidifies the membrane, but prevents crystallization (Bretscher and Raff, 1975). In a highly ordered pure lipid bilayer, the introduction of cholesterol can promote disorder and increase fluidity, since by binding to the phospholipids, it increases the spacing between them (Van Blitterswijk et al., 1981). Since under physiological conditions lipids are fluid, cholesterol acts as a lipid rigidifier (Shintzky, 1984). Removal of cholesterol from mouse lymphocyte membranes increased fluidity, and fluidity decreased with replacement of the cholesterol (Karnovsky et al., 1982). The cholesterol : phospholipid ratio is better correlated to membrane fluidity than total membrane cholesterol content (Cooper, 1978; Langlais and Roberts, 1985). As cholesterol : phospholipid ratio increases, fluidity decreases.

The length of hydrocarbon chains also affects membrane fluidity. As chain length increases, fluidity decreases (Gurr and James, 1980; Silver, 1985). The straight-line configuration of saturated fatty acids permits close, tight packing; long hydrocarbon chains must be highly ordered to fit within a membrane. The introduction of a double bond into even a very long hydrocarbon chain causes a marked increase in fluidity due to the mobility of the carbon atoms around that bond (Shinitzky, 1984). The double bond has the greatest

effect when placed into a fully saturated chain; this increase in fluidity becomes less pronounced as more double bonds are added (Shinitzky, 1984). Hydrocarbon chains featuring cis double bonds have lower transition temperatures than those with trans double bonds (Lee and Chapman, 1987).

Proteins also modulate membrane fluidity. Their bulk and rigidity tend to order the membrane (Shinitzky, 1984). A lipid molecule adjacent to a protein has limited "jump" positions due to the presence of the protein (Kleinfeld, 1987). As well, the rotational mobility of lipid double bonds is restricted by proteins (Kleinfeld, 1987). As protein concentration increases relative to lipid content in a membrane, some lipids will be trapped within the protein aggregates and will be further immobilized (Kleinfeld, 1987). However, phospholipid acyl chain order decreases in response to protein incorporation, possibly reflecting an influence of the uneven protein surface on the packing of the surrounding acyl chains (Kleinfeld, 1987). Proteins, therefore, interrupt lipid crystallization patterns and prevent total rigidification of the membrane while they increase membrane order (Shinitzky, 1984).

Calcium ions also affect membrane fluidity (Karnovsky et al., 1982; Shinitzky, 1984). Influx of extracellular Ca^{2+} can be a passive event (Clegg, 1983), but is also facilitated by the presence of a Ca^{2+}-binding protein, calmodulin (Casale et al., 1986), or a Ca^{2+} adenosine triphosphatase (ATPase) pump

(Ashraf et al., 1984). Most membrane phospholipids are negatively charged and are susceptible to crosslinking by Ca^{2+}, resulting in structural rearrangement of the phospholipids and a shift in overall membrane fluidity (Shinitzky, 1984). Ca^{2+} crosslinking with membrane proteins also causes conformational changes in the proteins, thus altering fluidity of the membrane (Karnovsky et al., 1982). The fluidizing effects of Ca^{2+} are well documented (Yanagimachi, 1981; Saxena et al., 1986; Wolf et al., 1986; Blumenthal, 1987; Kleinfeld, 1987), and have been associated with membrane instability (Blumenthal, 1987). Calcium-activated phospholipase action may lead to an accummulation of anionic phospholipids which results in fluid domains within the membrane, thus increasing the lateral mobility of the bilayer (Flechon et al., 1986; Fraser and Ahuja, 1988). Membrane proteins, therefore, have increased diffusibility within these highly fluid areas, further increasing the structural instability of these regions (Flechon et al., 1986). Lateral phase separations occur when the membrane bilayer develops into mixed gel and fluid phase regions due to any destabilizing factor which promotes the disordering of the phospholipid acyl chains (Kleinfeld, 1987). Calcium causes bilayer defects by inducing lateral phase separations, resulting in the formation of rigid crystalline domains of acidic phospholipids within a mixed membrane; such behaviour is correlated with increased membrane permeability

(Papahadjopoulous, 1978 in Blumenthal, 1987).

Ambient temperature affects the physical state of membrane phospholipids. In a model membrane consisting of a single type of phospholipid, the bilayer would undergo an abrupt phase change at that lipid's melting point, which is the phase transition temperature (Pringle and Chapman, 1981). Above the phase transition temperature, the lipid molecules are in a fluid state and the membrane is highly disordered (Lee, 1983). Below the phase transition temperature, rigidification of the phospholipid molecules occurs and membrane fluidity decreases (Shinitzky, 1984; Lee and Chapman, 1987).

The melting points of phospholipids are affected by the acyl side chains, increasing with increasing hydrocarbon chain length and decreasing rapidly with unsaturation (Stryer, 1981). Biological membranes contain a wide variety of phospholipids with varied chain lengths, varied degrees of unsaturation, and different head groups. The transition temperature for a biological membrane is broad, indicating the range of phase transition temperatures and the relative concentrations of the component lipids. Mixed phospholipid systems undergo lateral phase separations (Chapman et al., 1974) as the temperature decreases, causing clustering of the lipids in gel and fluid phases within the bilayer, reflecting the transition temperatures of the phospholipids.

Because of the redistribution and solidification of

membrane phospholipids at low temperatures, mobility of the protein molecules is severely inhibited. Lipids below the transition temperature squeeze the proteins out of the gel phase lattice, resulting in protein segregation into the remaining fluid areas (Chapman, 1983; Lee and Chapman, 1987). This passive clustering of the protein molecules is called "patch formation." In 1973, Speth and Wunderlich observed that patch formation induced by cooling was reversible with reheating. They postulated that with reheating above the transition temperature, the lipids fluidized and the aggregated proteins redistributed normally.

Morris and Clarke (1986) observed that as the environmental temperature of green algae (Chlamydomonas reinhardi) was reduced from 25-5C, the cellular uptake of K^+ decreased linearly with the decrease in temperature. The K^+ pump is mediated by an ATPase-driven transport protein in the membrane. They hypothesized that ATPase was rendered inactive at low temperatures, resulting in the failure of the K^+ pump. Hoffman et al. (1980) reported that as gel phase lipid containing protein fluidized, dramatic increases in the rotational mobility, reflex intensity, and enzyme activity occurred in sarcoplasmic reticulum cultures. Robertson and Watson (1986) demonstrated that cooled ram spermatozoa are unable to control calcium influx below 18-20C, suggesting the membrane response to cooling alters its normal permeability. In the same study, calcium regulation was restored with

rewarming only if the cooling was carried out slowly. Thus, permanent structural rearrangement due to rapid cooling does occur in membranes. It has been demonstrated, however, that particle clustering (patch formation) of spermatozoa membranes is only partially reversible following cooling and reheating (Holt and North, 1984; Canvin and Buhr, 1988). These observations show that the rotation and enzymatic activities of a protein are related (Pringle and Chapman, 1981), suggesting that the structure of a membrane affects its functional ability.

1.3.2 Capacitation

During testicular and epididymal maturation and ejaculation, the HPM acquires a series of tightly bound components, including sialoglycoproteins (Gordon et al., 1974; Nicholson et al., 1977; Voglmayr and Sawyer, 1986) and sterol sulfates (Legault et al., 1979; Bouthillier et al., 1984) which increase the net negative charge of the HPM (Yanagimachi, 1981; Orgebin-Crist and Oleson, 1984; Russell et al., 1984; Langlais and Roberts, 1985). Yanagimachi (1981) suggested capacitation involves the removal or alteration of decapacitation factors from the HPM surface. Decapacitation factors may act by stabilizing the HPM and preventing the acrosome reaction (Rogers and Bentwood, 1982). Sterol sulfates are membrane stabilizers that protect against osmotic shock (Bleau et al., 1974), suppress phase transitions of

phospholipids (Le Grimellec et al., 1984), and inhibit membrane cholesterol efflux (Langlais et al., 1981). Capacitation is associated with a decrease in the net negative surface charge of spermatozoa (Rosado et al., 1973; Vaidya et al., 1971) resulting from the removal of residues of neuraminic acid (sialoglycoprotein; Farooqui, 1983) and sterol sulfate (Langlais et al., 1981; Fayrer-Hosken et al., 1987) by neuramidases and sterol sulfatases present in the female tract (Farooqui, 1983; Langlais and Roberts, 1985). The reduction in HPM net negative surface charge by removal of the decapacitation factors causes the destabilization of the HPM which is associated with capacitation (Farooqui, 1983).

HPM cholesterol is also involved in capacitation; sterol acceptors are present in uterine, oviductal, and follicular fluids (Langlais et al., 1981; Davis, 1981, 1982). A low membrane cholesterol : phospholipid ratio destablizes membranes (Le Grimellec et al., 1984), and the removal of cholesterol from bovine spermatozoa predisposed the cell to undergo the acrosome reaction in response to a lysophosphatidylcholine challenge (Ehrenwald et al., 1988; Fayrer-Hosken et al., 1987). Exchange of cholesterol between the HPM and extracellular regions is mediated by serum levels of lipoproteins, apolipoproteins, albumin, and lecithin-cholesterol transferase (Langlais and Roberts, 1985). Estrogens promote synthesis of cholesterol carrier proteins in the female (Langlais and Roberts, 1985). Thus, at

ovulation, when the female's estrogen concentrations peak near ovulation, maximum levels of these cholesterol carrier proteins are present to facilitate cholesterol efflux from the spermatozoa HPM and to promote capacitation which is essential for fertilization (Langlais and Roberts, 1985).

The HPM changes associated with capacitation cause membrane instability and an increase in fluidity which may increase calcium permeability of the membrane (Clegg, 1983; Langlais and Roberts, 1985). The influx of Ca^{2+} stimulates the acrosome reaction.

1.3.3 The Acrosome Reaction

Once capacitation has occurred, the destabilized HPM allows a passive influx of extracellular Ca^{2+} through the HPM (Clegg, 1983; Nikolopoulou et al., 1986; Thomas and Meizel, 1988). This Ca^{2+} neutralizes negative charges on the inner surface of the HPM and on the OAM eliminating any repulsion between these membranes and facilitating their apposition (Fraser, 1984). Davis (1978 in Yanagimachi, 1981) hypothesized that intracellular Ca^{2+} binds to the polar heads of the phospholipids of the HPM and OAM, creating an ionic bridge, thus forcing the close proximity of the membranes. Indeed, specific Ca^{2+} binding sites between the HPM and OAM have been identified (Watson and Plummer, 1986; Ruknudin et al., 1988). An increase in Ca^{2+} induced a phase transition from the liquid to gel state and the formation of separate

phase microdomains along the HPM (Yanagimachi, 1981; Scandella et al., 1982; Wolf et al., 1986).

A more recent theory regarding the role of Ca^{2+} in the acrosome reaction involves phospholipase A (Clegg, 1983; Langlais and Roberts, 1985) which catalyses the hydrolysis of phospholipids into lysophospholipids and free fatty acids (Langlais and Roberts, 1985). Phospholipase cleavage can also produce the phosphate moiety and a diacylglycerol. Diacylglycerol in the presence of ATP can produce the phosphatidic acid, a fusogen (Fraser and Ahuja, 1988). Nikolopoulou et al. (1986) observed that the HPM from acrosome-reacted boar spermatozoa contained a higher percentage of diacylglycerols and free fatty acids than membranes from non-reacted spermatozoa, suggesting phospholipase action. These products of phospholipase activity can increase membrane fluidity, and induce membrane instability and fusion (Karnovsky et al., 1982).

The influences of Ca^{2+}, therefore, include the establishment of crosslinked negative charges (Karnovsky et al., 1982; Shinitzky, 1984) that create an ionic bridge between molecules (Yanagimachi, 1981; Shinitzky, 1984). Furthermore, Ca^{2+} serves as a catalyst in phospholipase reactions (Langlais and Roberts, 1985). All these events promote membrane destabilization. The acrosome reaction appears to be specifically Ca^{2+} dependent as other divalent cations such as Zn^{2+} and Mg^{2+} do not satisfactorily replace Ca^{2+}

during fertilization (Fraser, 1984).

1.4 MODULATORS OF BOAR HPM FUNCTION

The hypothesis of this study states that cryo-induced ultrastructural changes occur in HPM that interfere with capacitation and/or the acrosome reaction. These changes alter membrane fluidity and spermatozoa response to calcium in the presence of membrane modulators.

Chapter II

MATERIALS AND METHODS

2.1 OUTLINE OF EXPERIMENTS

There were five experiments performed (table 1). The first four involved the separation of the head plasma membranes (HPM) from the sperm body membranes (SBM). Six trials were performed in Experiment One, to examine the effects of commercial freezing and thawing on the fluorescence polarization values of SBM and HPM in the absence and presence of 10mM Ca^{2+}. Five trials in Experiment Two tested the fluidity of HPM following cold shock of intact spermatozoa, or the dose effects of two levels of phospholipase A_2 from either snake venom (Crotalus adamanteus) or from bee venom (Apis mellifer; Sigma Chemical Co., St. Louis, MO) on fluorescence polarization values of HPM from normal spermatozoa. For Experiment Three, five trials were used to obtain the effects of three doses of defatted bovine serum albumin (dBSA) (Sigma Chemical Co.) on the fluorescence polarization values of either snake venom or bee venom phospholipase A_2-containing HPM samples. For Experiment Four, two molecular probes, trans-parinaric acid (t-PnA) and cis-

two molecular probes, trans-parinaric acid (t-PnA) and cis-parinaric acid (c-PnA; Molecular Probes Inc., Eugene, OR) were used to monitor the viscosity changes of the HPM throughout six trials; t-PnA was the probe used in Experiments One, Two, and Three. The six trials of Experiment Five measured the Ca^{2+} content of whole sperm treated with either cold shock or four doses each of snake venom phospholipase A_2 or bee venom phospholipase A_2. In each trial of each Experiment, all treatments were applied to identical aliquots of membrane or spermatozoa sample.

2.2 SEMEN COLLECTION

For all Experiments, semen was collected by the gloved hand technique, once or twice weekly from Managra cross or Yorkshire boars of proven fertility. Semen was fractionated visually into sperm-rich and sperm-poor portions during collection, and the sperm-poor semen was discarded. The sperm-rich fraction was collected into a warmed 500 mL insulated vacuum flask; the opening was covered with a double layer of J-Cloth (Johnson and Johnson Inc., Toronto) to filter the gel particles. For Experiments One, Two, Three, and Four, at least two ejaculates from different boars were combined together to generate membrane samples for each trial. In Experiment Five, the six trials were performed with ejaculates from different boars, with a single ejaculate used per trial.

Table 1. Summary of experiments.

EXPT.(n)	TREATMENT	PROCEDURE
1 (6)	Cooled HPM ± 10 mM Ca^{2+} Cooled SBM ± 10 mM Ca^{2+} Thawed HPM ± 10 mM Ca^{2+} Thawed SBM ± 10 mM Ca^{2+}	fluorescence polarization (t-PnA)
2 (5)	HPM + 10 mM Ca^{2+} (Control) Control + 0.01 μg pA_2 (SV)*/mL Control + 0.0001 μg pA_2 (SV)/mL Control + 0.01 μg pA_2 (BV)**/mL Control + 0.0001 μg pA_2 (BV)/mL Cold shocked	as expt.1
3 (5)	HPM + 10 mM Ca^{2+} + 0.0001 μg pA_2 (BV)/mL (Control a) Control a + 1 mg dBSA/mL Control a + 0.01 mg dBSA/mL Control a + 0.0001 mg dBSA/mL HPM + 10mM Ca^{2+} + 0.0001 μg pA_2 (SV)/mL (Control b) Control b + 1 mg dBSA/mL Control b + 0.01 mg dBSA/mL Control b + 0.0001 mg dBSA/mL	as expt.1
4 (6)	HPM with t-PnA (Control c) Control c + 10mM Ca^{2+} HPM with c-PnA (Control d) Control d + 10mM Ca^{2+}	fluorescence polarization (t-PnA or c-PnA)
5 (6)	Whole sperm (Control) Control + 1 μg pA_2 (BV)/mL Control + 0.01 μg pA_2 (BV)/mL Control + 0.001 μg pA_2 (BV)/mL Control + 0.0001 μg pA_2 (BV)/mL Control + 1 μg pA_2 (SV)/mL Control + 0.01 μg pA_2(SV)/mL Control + 0.001 μg pA_2(SV)/mL Control + 0.0001 μg pA_2(SV)/mL Cold shocked	$^{45}Ca^{2+}$radioassay

* snake venom source of phospholipase A_2
** bee venom source of phospholipase A_2

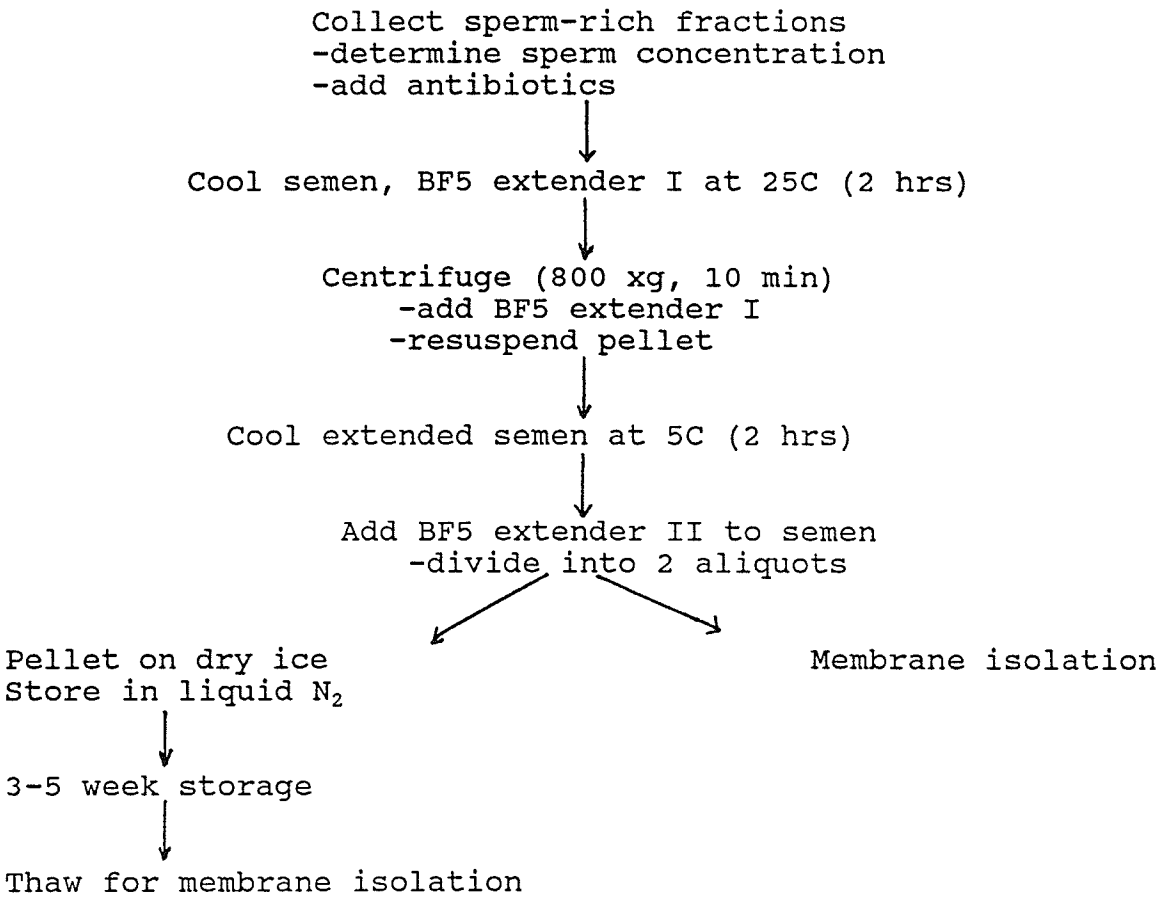
2.3 FREEZING AND THAWING

For Experiment One, semen was prepared for freezing and thawing by accepted industry procedures (variation from Pursel and Johnson, 1975; figure 1). The spermatozoa concentrations of the ejaculates were measured using a calibrated spectrophotometer. Antibiotics (Lincospectin dihydrostreptomycin) were added at a dose of 0.3 mL per 50 mL of raw semen. The semen was allowed to cool at room temperature in the insulated vacuum flask about two hours from collection time until it reached approximately 25C. The BF5 extender fraction I (appendix 1), which had been previously warmed to 37C, was also held in a vacuum flask and cooled at room temperature to 25C. The possible number of tubes of frozen semen each containing 7 billion spermatozoa per ejaculate was calculated by:

$$\frac{(\text{sperm-rich concentration})(\text{ejaculate volume})}{7 \text{ billion spermatozoa/tube}} = \text{doses of frozen semen}$$

The 25C semen was centrifuged in 250 mL bottles (800xg, 25C, 10 minutes). The supernatant was discarded and BF5 extender fraction I was added to the pellet (5 mL per calculated dose). The pellets were resuspended by pipette and cooled to 7-5C by submersing the 250 mL bottles into 600 mL beakers filled with 200 mL of 25C water and holding them at 5C for 2 hours. At this time, glycerol-containing BF5 extender fraction II

Figure 1: Outline of freezing protocol.



(appendix 1), also at 7-5C, was added. The semen was divided into two aliquots. The first aliquot (cooled fraction) was used immediately for the membrane isolation procedure; the second aliquot (thawed fraction) was dropped by syringe into depressions of 100 μL-200 μL volume on dry ice slabs and allowed to freeze for approximately 3 minutes. The frozen semen pellets were dropped into storage tubes and held in liquid nitrogen for three to five weeks.

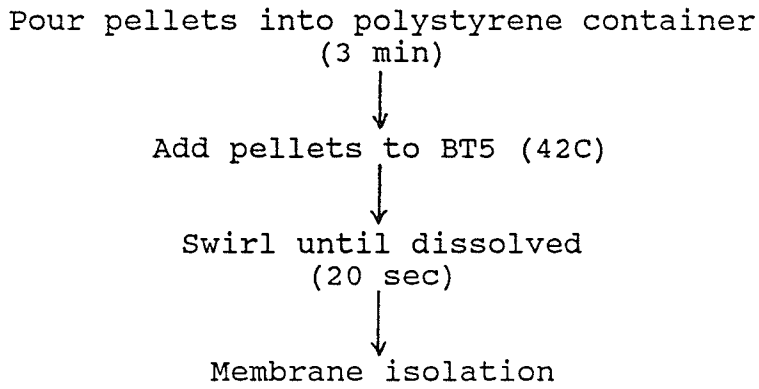
They were then thawed by accepted commercial procedure (Pursel and Johnson, 1975; figure 2) and the plasma membranes isolated. For thawing, all materials including glassware and Beltsville Thawing Solution (BTS; appendix 2) were warmed to 38C. For each semen tube, 60 mL BTS were poured into a 250 mL beaker and warmed to 42C. The pellets were placed in an empty polystyrene container for three minutes. They then were added to the warm BTS and swirled until completely thawed (about 20 seconds). The thawed fraction was ready for the membrane isolation procedure at this point.

2.4 MEMBRANE ISOLATION

Membrane isolation procedures for Experiments One and Four were carried out at 25C while for Experiments Two and Three temperature was maintained at 30C.

In Experiment One, the thawed fraction was poured into 250 mL bottles and centrifuged (2500xg, 25C, 10 minutes). The

Figure 2: Outline of the thawing procedure.



supernatants were aspirated, reserving enough to resuspend the pellets to a total volume of 150 mL. For Experiments Two, Three, and Four, the sperm-rich fraction was filtered twice through a double layer of Miracloth (Calbiochem, La Jolla, CA) into a flask in a waterbath held at the appropriate temperature. In Experiment Two, the sperm-rich fraction was divided in half and one aliquot (the cold shocked sample) was diluted approximately ten-fold with 30C Buffer 1 (5 mM Tris chloride, 0.25 M sucrose, pH 7.4), poured into a 6 L Erlenmeyer flask and held in an ice bath at 2C for 30 minutes. Following this cold shock treatment, the diluted semen was centrifuged (650xg, 30C, 10 minutes). The supernatants were aspirated, reserving a sufficient volume to resuspend the pellets to a total of 150 mL. Throughout Experiment Two, the cold shocked sample was prepared identically to, but held separately from, the nontreated semen. Beyond this point, membrane isolation procedures for all samples and Experiments were identical (figure 3).

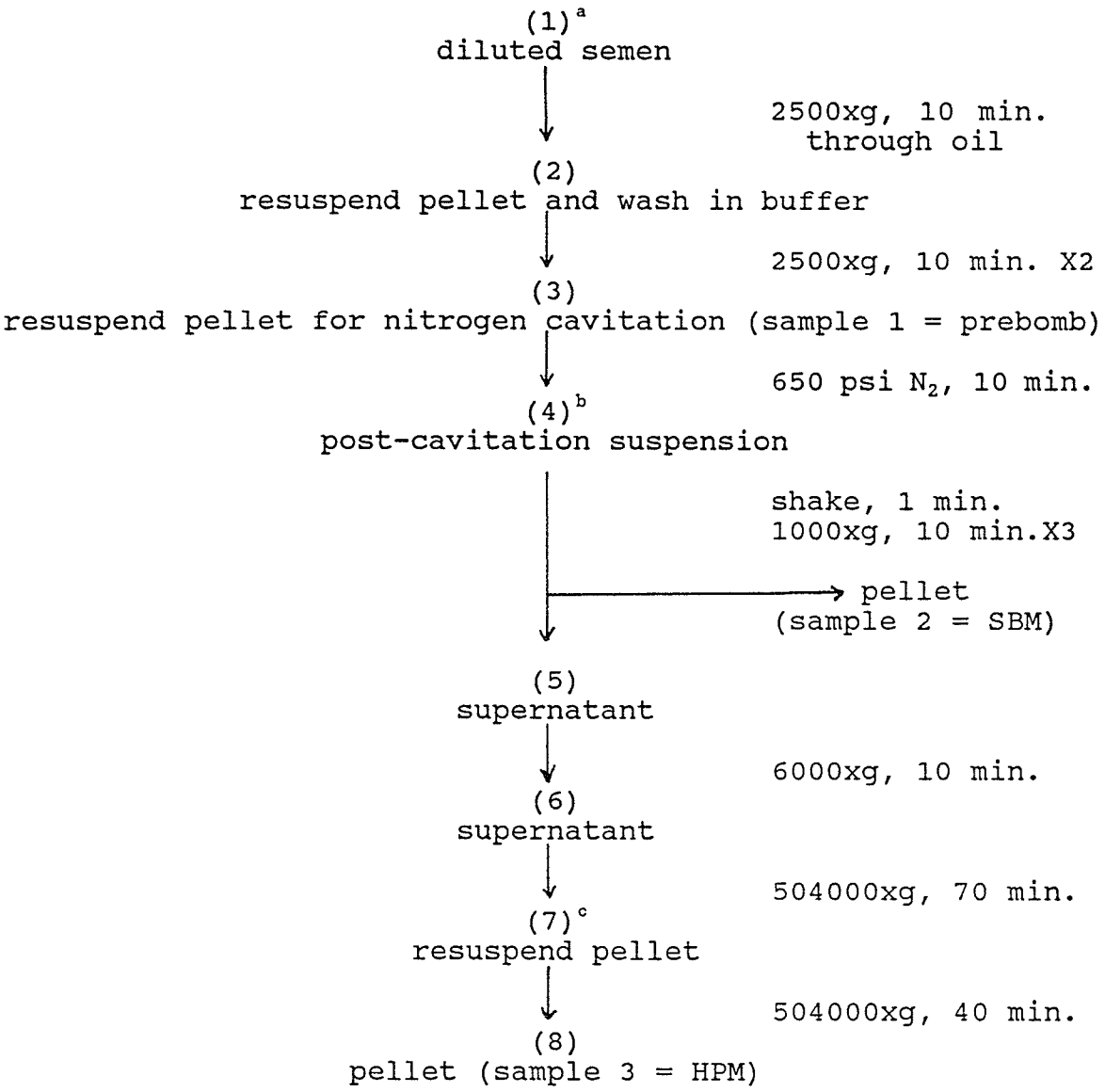
Two highly refined silicon-based oils, 550 and 1107 fluids (Dow Corning, Mississauga, ON), were combined 1:1 (v/v), hand shaken, centrifuged (1500xg, 15 minutes) and the supernatant harvested. The semen was diluted 1:1 with Buffer 1 and layered 1:1 (v/v) on the oil supernatant (25 mL oil per test tube). The semen-oil gradient was centrifuged (2500xg, 10 minutes). The gel/seminal fluid/oil supernatant was discarded, and the sperm pellet was resuspended with an

original volume of Buffer 1 (50 mL). This was centrifuged and resuspended twice more to remove any residual oil. The final pellet was resuspended in Buffer 1 to 31 mL and poured into a Parr cell cavitation unit and a 1 mL aliquot was removed and frozen for protein and enzyme analyses (prebomb sample).

Nitrogen was added to the Parr cavitation unit, obtaining a pressure of 650 psi over a 90 second interval and held for 10 minutes (Gillis et al., 1978) to allow nitrogen gas to infiltrate the spermatozoa. The spermatozoa suspension was slowly extruded from the cavitation unit over a 90 second interval into an equal volume (30 mL) of Buffer 2 (5 mM Tris chloride, 0.25 M sucrose, pH 5.0). This cavitate was vigorously hand shaken for one minute to facilitate removal of the HPM (Kaplan et al., 1984). The shaken cavitate was divided into two 50 mL tubes which were then filled with Buffer 2 and centifuged three times (1000xg, 10 minutes). One mL samples from the final pellet (SBM) were collected and frozen for protein and enzyme analyses in Experiment One.

The pooled supernatants were centrifuged (6000xg, 10 minutes). The resultant pellets were discarded and the supernatants, which contained the HPM, were pipetted into quickseal ultracentrifuge tubes (Beckman Instruments (Canada) Inc., Burnaby, BC) and centrifuged (504000xg, 70 minutes). The supernatants were discarded and the HPM pellets were resuspended in Buffer 3 (10 mM Tris, 0.9% sodium chloride, pH 7.4) and transferred to one tube and centrifuged (504000xg,

Figure 3: Membrane isolation procedure.



[a] steps 1-3 use Buffer 1
[b] steps 4-6 use Buffer 2
[c] steps 7-8 use Buffer 3

40 minutes). The resultant pellet of HPM was resuspended in 3 mL of Buffer 3 and homogenized by hand in a glass-teflon tissue homogenizer with approximately 10-12 strokes of the pestle. This final HPM sample was reserved for fluorescence polarization, protein, and enzyme analyses.

The final protein concentrations of the HPM, SBM, and the prebomb samples were determined (Bradford, 1976) using bovine serum gamma globulin (Sigma Chemical Co.) as a standard.

2.5 FLUORESCENCE POLARIZATION

For Experiments One, Two, and Three, fluorescence polarization values were obtained on a LS5 Perkin-Elmer spectrofluorimeter fitted with a polarizing accessory (Perkin-Elmer, Oak Brook, IL) with excitation and emission slit widths set at 5 and 10 nm respectively. An SLM 8000 polarizing spectrofluorimeter (SLM Instruments, Inc., Urbana, IL) was used to measure fluorescence for Experiment Four. Stock solutions of t-PnA and c-PnA (2mM in pharmaceutical grade ethanol) were stored dark under nitrogen gas at -20C and replaced every three months. For each analysis, the stock solution was diluted 1000-fold in Buffer 3 and 1500 μL of this diluted probe solution was added to 1500 μL of membrane (100 μg protein/mL) in a quartz cuvette. Final concentrations were 1 μM probe and 50 μg membrane protein per mL.

For the Ca^{2+}-containing samples, 10 μL of a 3 M calcium

chloride solution was added to each cuvette by pipette, giving a non-physiological Ca^{2+} concentration of 10 mM. The samples were allowed to incubate for about 10 minutes following the addition of Ca^{2+}.

For Experiments Two and Three, phospholipase A_2 stock solutions of 0.15 mg per mL from snake or bee venom were made up in Buffer 3 immediately before use each day, diluted to appropriate levels, and 20 μL added to the cuvettes. The specific activities of the snake and bee venom phospholipase A_2 were 230 and 1050 units per mg protein respectively. For Experiment Two, the final concentrations of phospholipase A_2 in the cuvettes were 10 or 0.1 ng per mL. For Experiment Three, 0.1 ng phospholipase per mL was chosen and tested against 1000, 10, or 0.1 μg of dBSA per mL.

The dBSA stock solution for Experiment Three was prepared weekly and stored dark at 5C. For Experiments Two and Three, fluorescence readings began immediately following the addition and mixing of the phospholipase A_2 and dBSA solutions.

The cuvette holder was connected to a Lauda RC3 circulating waterbath and the fluorescence measurements were taken constantly for 2.5 hours (Experiments One and Four) or two hours (Experiments Two and Three). The sample temperatures were maintained at 25C (Experiments One and Four) or 30C (Experiments Two and Three) and monitored by a thermocouple placed in a cuvette. The wavelengths allowing maximum excitation and emission intensities were determined

daily and were 327.2 ± 0.4 nm (mean ± S.E.) and 409.8 ± 10.7 respectively.

The fluorescence intensities were converted to polarization values using the Perrin equation:

$$\text{Polarization Value} = \frac{IVpara + IVperp \times G}{IVpara + IVperp \times G};$$

where:

$$G = \frac{IHpara}{IVperp}$$

I=intensity of the exciting light

V=vertically polarized exciting light

H=horizontally polarized exciting light

para=the emission light parallel to the vertical component

perp=the emission light perpendicular to the vertical component

2.6 ENZYME ANALYSIS

All samples collected during the Experiments for enzyme analysis were frozen immediately (-20C). Using the nitrophenylphosphate (Sigma Chemical Co.) method of Linhardt and Walter (1963), the activity of the HPM marker enzyme, alkaline phosphatase (Soucek and Vary, 1984) was determined. The specific activities of the samples were calculated in order to estimate the purity of the HPM sample.

2.7 ^{45}CALCIUM RADIOASSAY

For Experiment Five, HBGS diluent (20 mM HEPES-sodium hydroxide, 40 mM glucose, 125 mM sodium chloride, pH 7.0, 310 mOsm/kg) contained ^{45}Calcium ($^{45}Ca^{2+}$; specific activity 0.1 μCi/mL) as calcium chloride to a final Ca^{2+} concentration of 300 μM. In each of six trials the sperm rich fraction from one boar was divided into twenty identical aliquots and two aliquots were used per treatment. Eighteen aliquots were each extended ten fold in HBGS containing 0, 0.1, 0.001 or 0.0001 μg per mL phospholipase A_2 (Sigma Chemical Co.) from snake venom and the same concentrations of phospholipase A_2 from bee venom and incubated at 30C for 240 minutes. The remaining two aliquots represented the cold shocked sample. These aliquots were diluted with HBGS containing no phospholipase and held on ice for 240 minutes. Aliquots from all treatments were removed at time zero immediately after dilution and at 30 minute intervals thereafter. Calcium movement was terminated and the spermatozoa were prepared for scintillation counting by the method of Robertson and Watson (1986).

2.8 STATISTICAL ANALYSIS

The least square means of the initial polarization values of each trial within a treatment were analyzed for each Experiment by the General Linear Models procedure (GLM; SAS

Inc., 1985) for least significant differences among treatments and/or between membrane types. For analysis of the fluidity changes over time, the polarization values of each trial within an experiment were adjusted by subtracting the starting polarization value of each treatment from all subsequent polarization values within that treatment to remove any variation among trials due to differences among the initial values. The means of these adjusted values were analyzed by stepwise regression on a polynomial equation, using time and powers of time as dependant variables. For nonlinear data, the NLIN procedure (SAS Inc., 1985) was used to fit the linear segments and estimate the slopes and intercepts. Breakpoints on the x-axis were estimated by fitting the linear segments together, also using NLIN. Experiments One and Two were analysed by a factorial design testing for all possible interactions. The data for Experiments Two were analysed by multiple comparisons.

For the $^{45}Ca^{2+}$ radioassay, the duplicates of treatments were pooled within trials, the data were converted to nmol $Ca^{2+}/10^{9}$ spermatozoa and the least square means of the pooled data were analyzed by GLM for any significant differences among treatments within specific times, or within treatments over time.

Chapter III

RESULTS

3.1 EXPERIMENT ONE

Fluidity of cooled spermatozoa membranes (HPM and SBM) changed over time (figures 4 and 5), as did that of frozen-thawed membranes (figures 6 and 7). Freezing and thawing significantly affected fluidity parameters of both membranes types ($p = .0008$; table 2), and the changes all membranes underwent over time regardless of the inclusion of Ca^{2+} ($p = .0022$). The fluidity shifts of HPM over time differed significantly from those of SBM ($p = .0001$) regardless of the processing method. Ca^{2+} significantly affected all fluidity parameters ($p = .0076$). There was a significant interaction between membrane type and Ca^{2+} ($p = .0436$), and between time-related fluidity changes and Ca^{2+} (.0001). The HPM and SBM responded differently to Ca^{2+} over time ($p = .0001$).

The initial unadjusted polarization values of all samples were similar. There was no effect of the level of processing, membrane type or Ca^{2+} on the initial unadjusted polarization values.

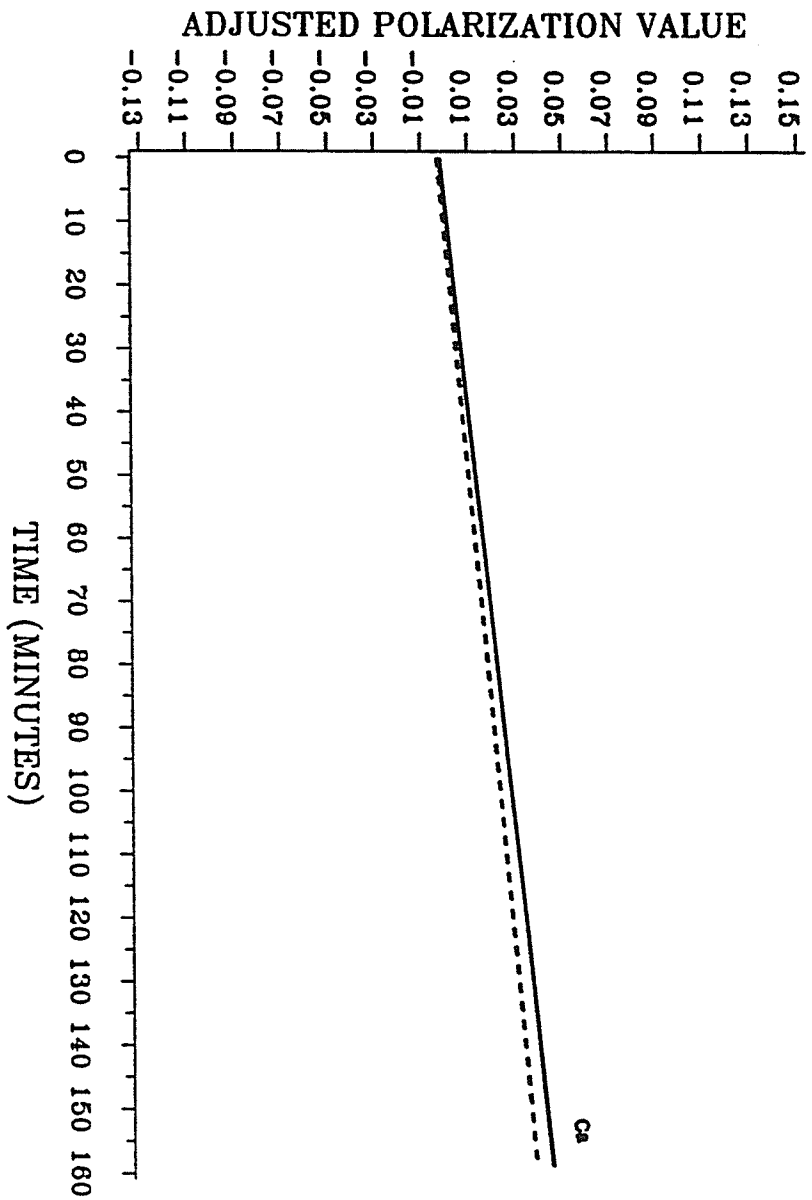


FIGURE 4: FLUIDITY OF COOLED HPM OVER TIME

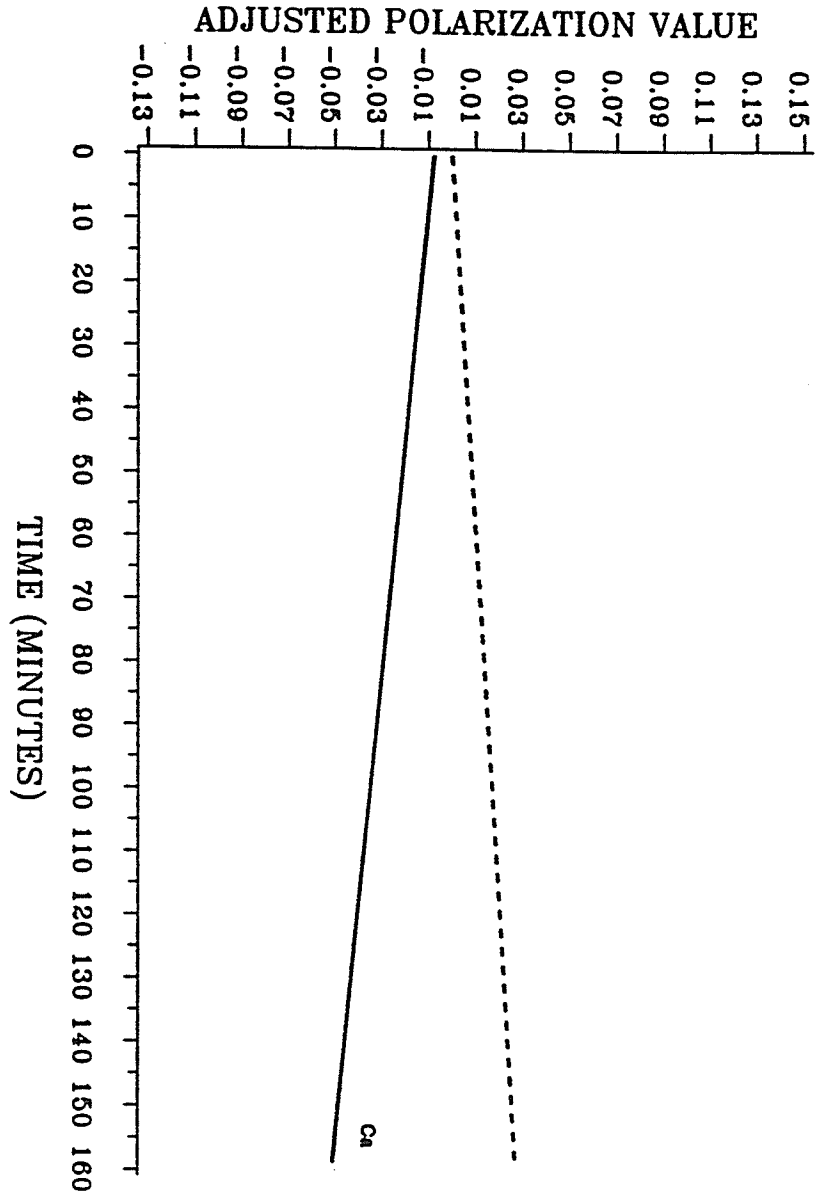


FIGURE 5: FLUIDITY OF COOLED SBM OVER TIME

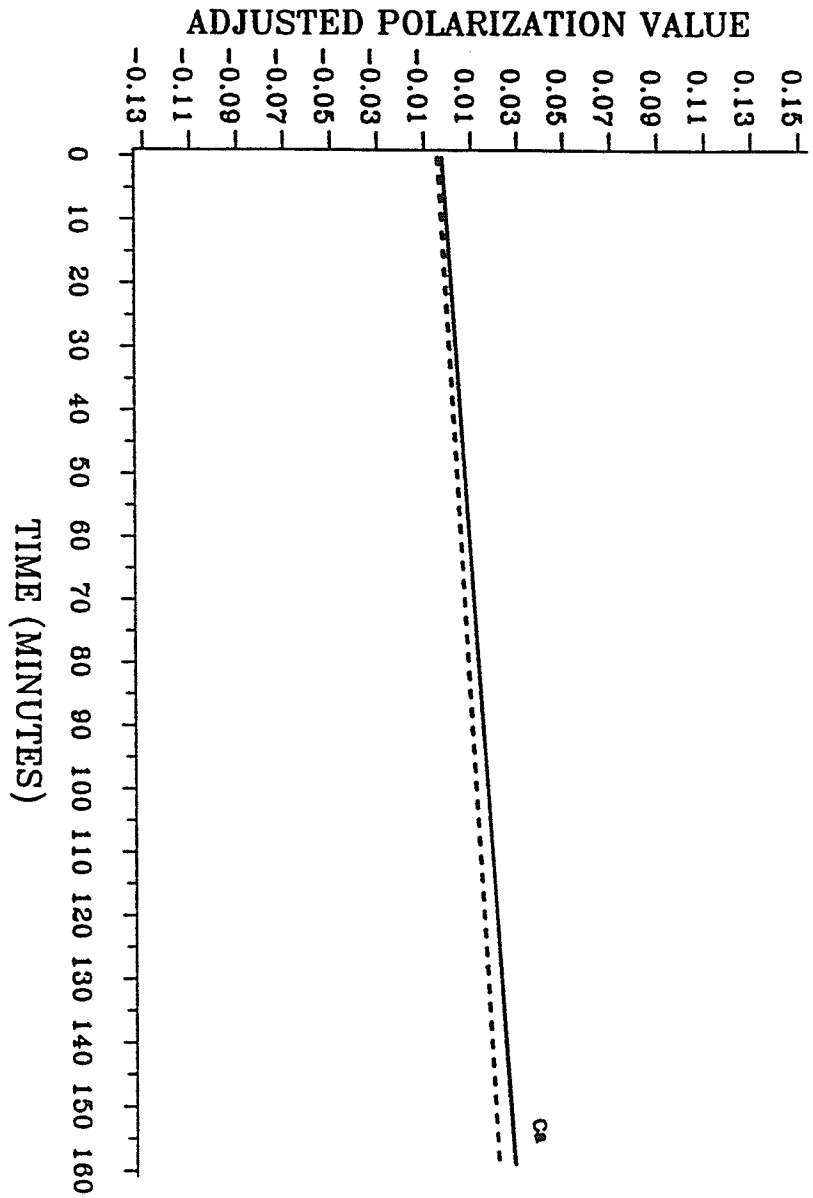


FIGURE 6: FLUIDITY OF THAWED HPM OVER TIME

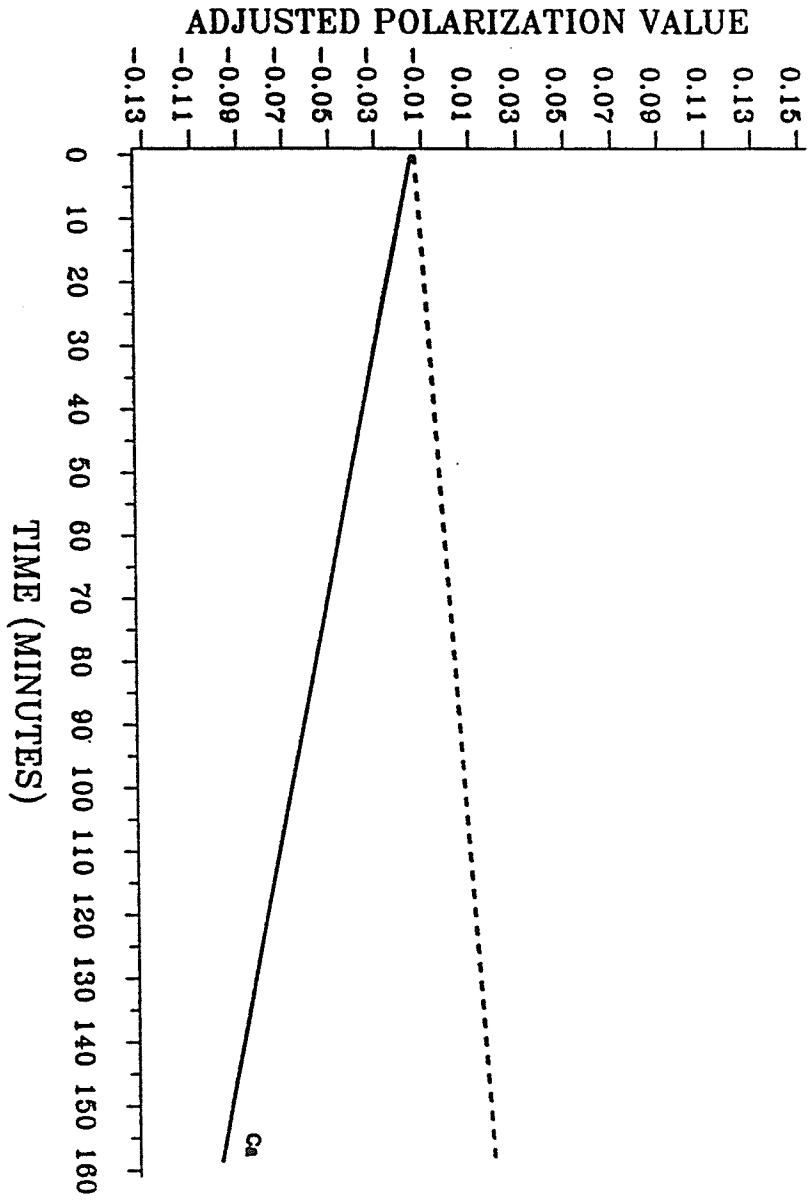


FIGURE 7: FLUIDITY OF THAWED SBM OVER TIME

Table 2: Effects of freezing and thawing on the fluidity of spermatozoa membranes.

Sample	Slope ± S.E. (PV/min x 10^5)	Model R^2
Cooled HPM	24.7 ± 2.8*	0.338
Cooled HPM + Ca^{2+}	25.7 ± 2.7	0.428
Cooled SBM	22.3 ± 8.6	0.043
Cooled SBM + Ca^{2+}	-39.4 ± 7.9	0.146
Thawed HPM	16.0 ± 2.2	0.263
Thawed HPM + Ca^{2+}	18.1 ± 2.6	0.239
Thawed SBM	18.8 ± 4.2	0.115
Thawed SBM + Ca^{2+}	-77.2 ± 6.7	0.461

* Statistically significant differences are described in the text.

3.2 EXPERIMENT TWO

Fluidity of the control HPM differed from the phospholipase A_2-containing and cold shocked HPM samples ($p = .039$; figures 8-11). Treatments also significantly affected time-related shifts in membrane fluidity ($p = .0041$). The HPM from the control spermatozoa differed from the cold shocked ($p = .0117$) which also changed differently over time ($p = .0051$). The control HPM underwent a linear decrease in fluidity, which differed from the slopes of the phospholipase A_2- treated HPM ($p = .0044$; table 3). HPM treated with phospholipase A_2 differed from HPM of cold shocked spermatozoa ($p = .0397$). Bee venom and snake venom sources of phospholipase A_2 caused different fluidity responses over time ($p = .0313$).

There were no differences among initial unadjusted polarization values of all samples.

3.3 EXPERIMENT THREE

The overall fluidities of HPM exposed to the two sources of phospholipase A_2 differed ($p = .0081$), however their relative changes over time were equivalent ($p = .2287$; tables 4 and 5). The level of dBSA significantly affected fluidity of HPM treated with phospholipase A_2 ($p = .0001$); again,

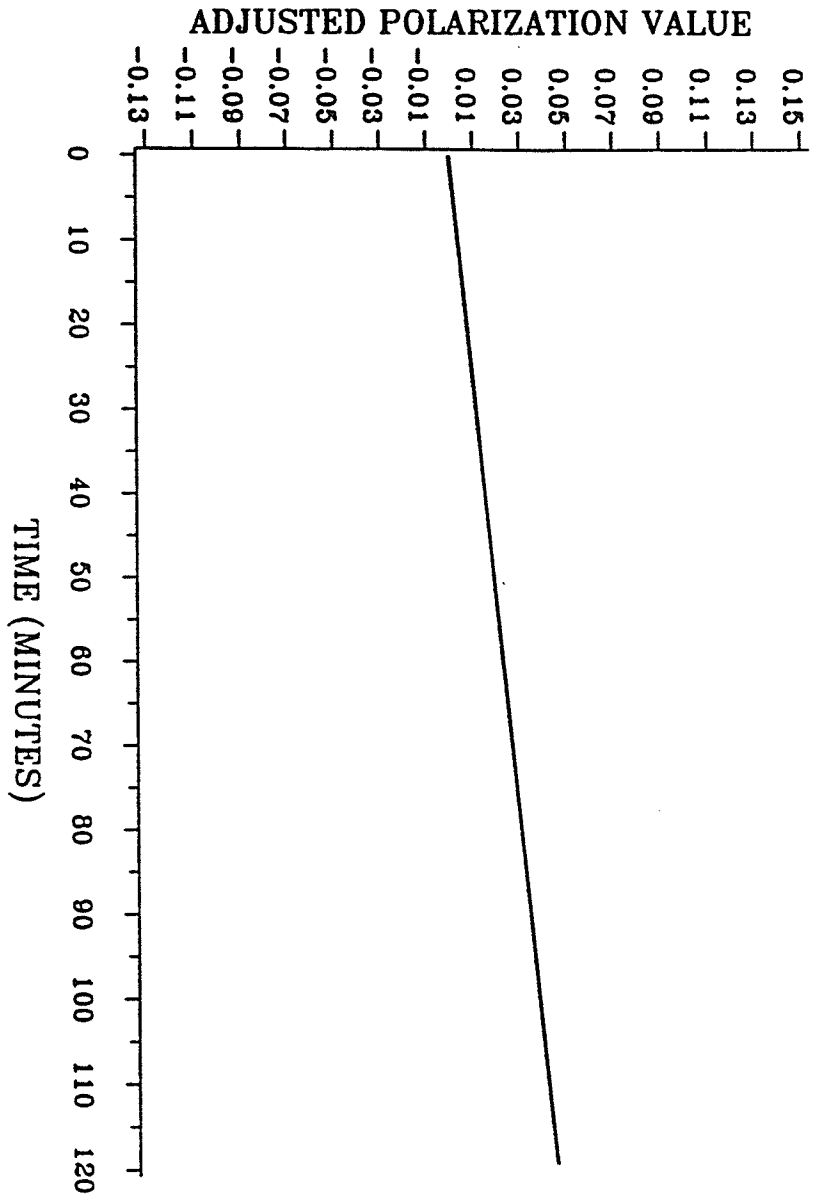
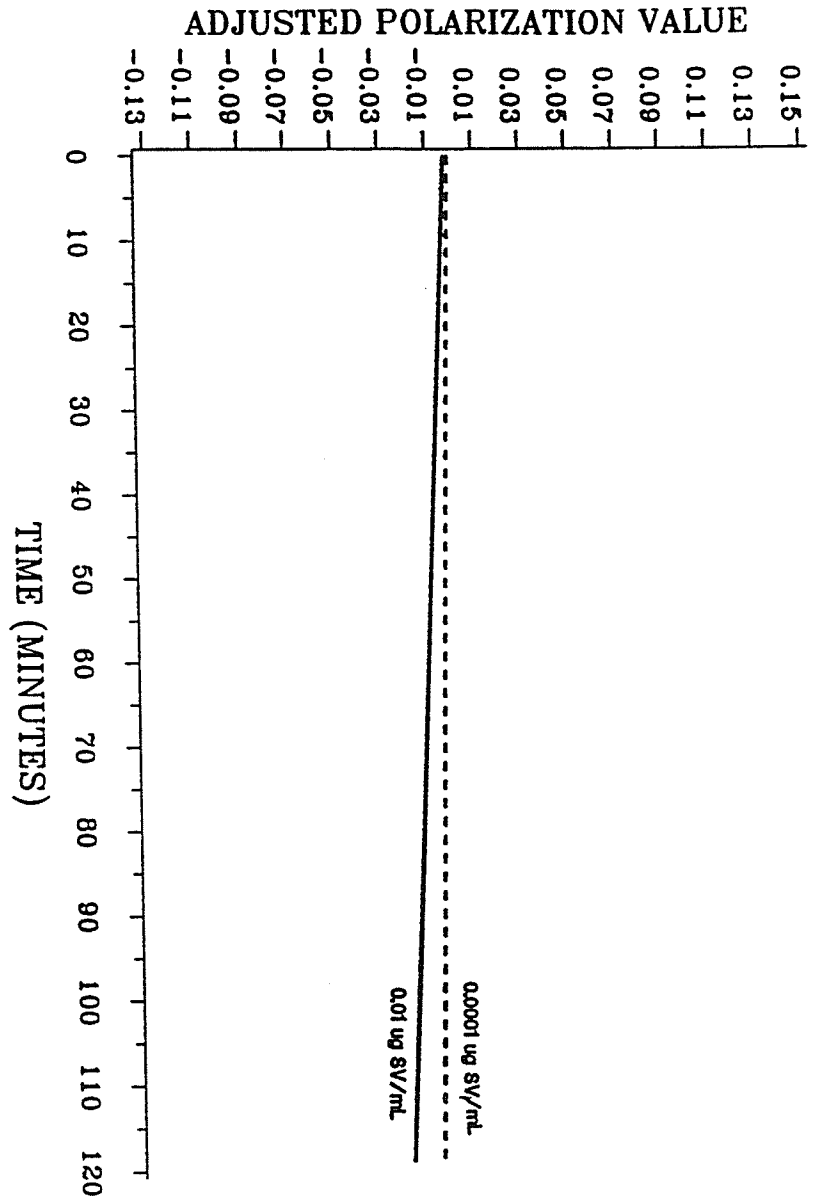


FIGURE 8: FLUIDITY OF CONTROL HPM OVER TIME

FIGURE 9: FLUIDITY OF HPM + SNAKE VENOM PHOSPHOLIPASE A_2 OVER TIME



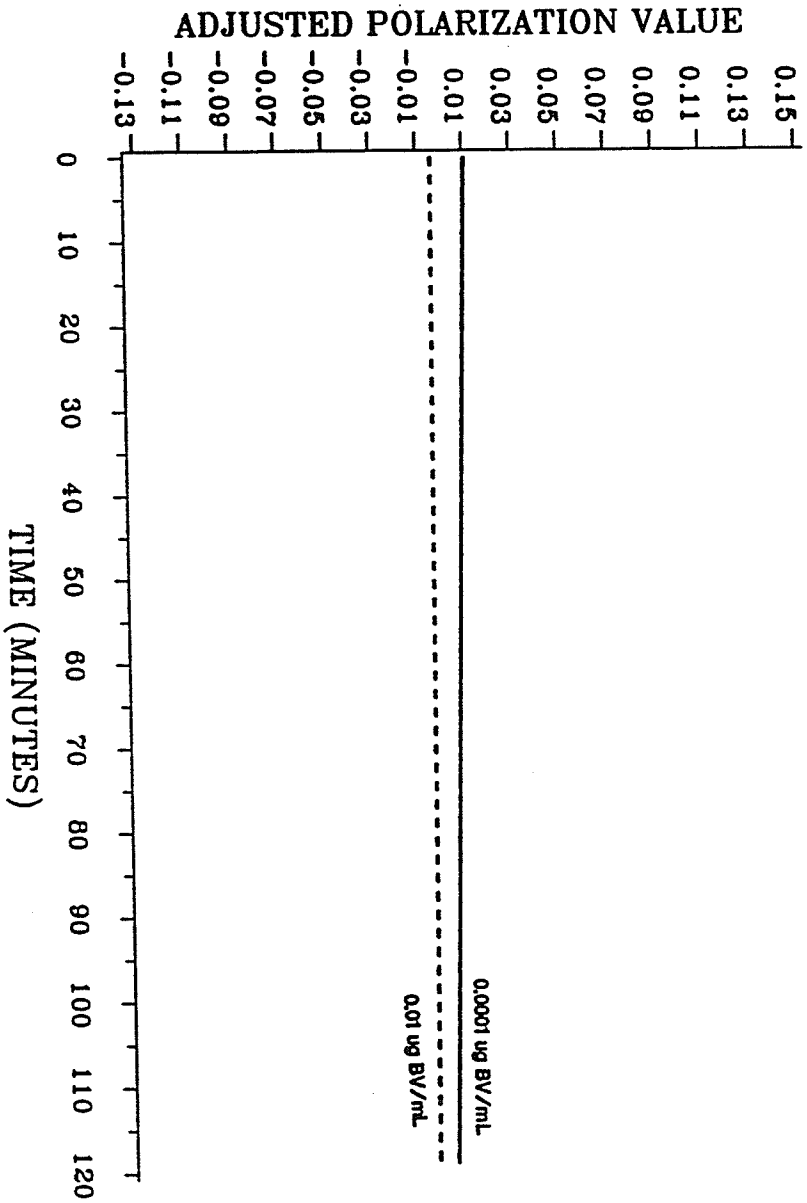


FIGURE 10: FLUIDITY OF HPM + BEE VENOM PHOSPHOLIPASE A_2 OVER TIME

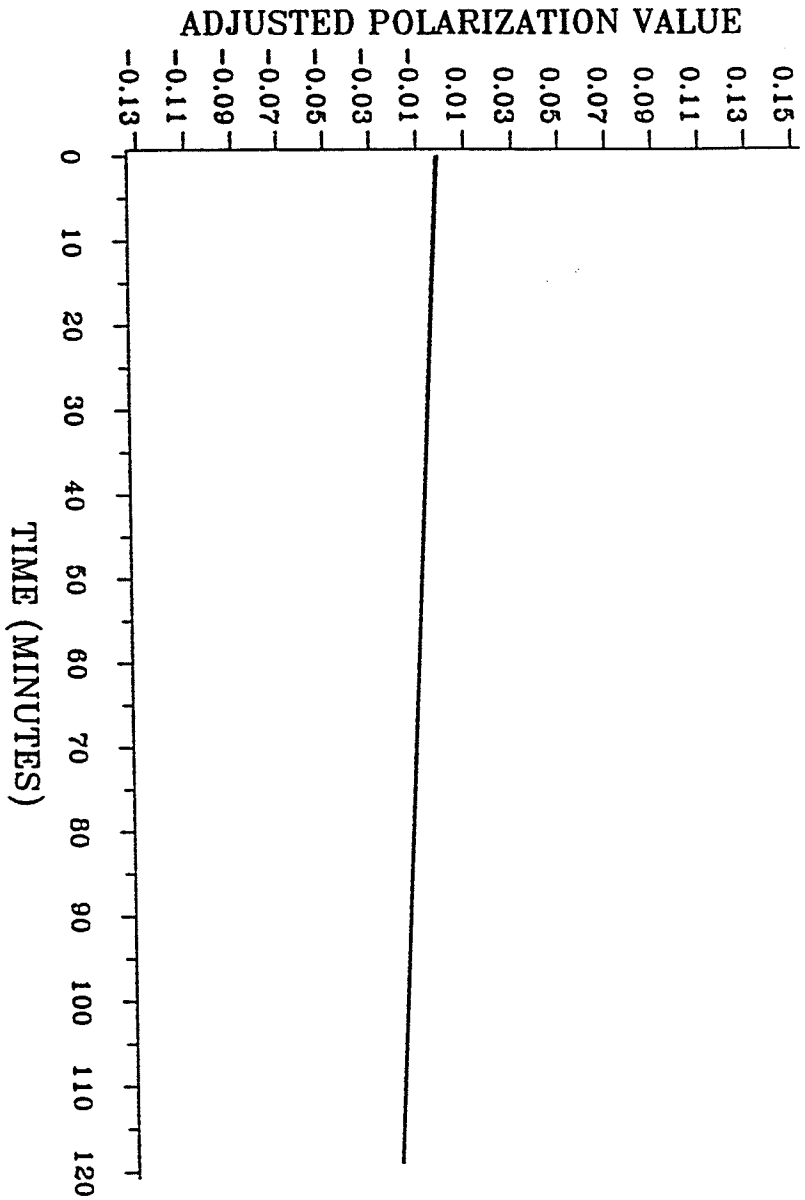


FIGURE 11: FLUIDITY OF COLD SHOCKED HPM OVER TIME

Table 3: Effect of phospholipase A_2 from snake or bee venom and cold shock on HPM fluidity.

Treatment	Slope ± S.E. (PV/min x 10^5)	Model R^2
Control	37.2 ± 5.0*	0.16321
0.01 μg phospholipase A_2 (SV)/mL	-7.6 ± 5.0	0.01458
0.0001 μg phospholipase A_2 (SV)/mL	-15.0 ± 5.3	0.04963
0.01 μg phospholipase A_2 (BV)/mL	-2.5 ± 5.1	0.00128
0.0001 μg phospholipase A_2 (BV)/mL	-3.9 ± 7.6	0.00218
Cold shocked	-17.2 ± 6.7	0.04994

* Statistically significant differences are described in the text.

SV = phospholipase A_2 from snake venom

BV = phospholipase A_2 from bee venom

Table 4: Fluidity slopes of HPM with phospholipase A_2 from bee venom with or without dBSA.

Concentration of dBSA (mg/mL)	Slope ± S.E. (PV/min x 10^5)	Model R^2
0.0 Control*	18.6 ± 7.0[a]	0.06876
0.0001	7.4 ± 7.9[a]	0.00080
0.01	-9.0 ± 7.5[a]	0.00267
1.0	-12.0 ± 3.5[b]	0.09708

* All samples contained 0.0001 μg/mL phospholipase A_2 from bee venom.

[a] Concentration of dBSA affected the slope ($p = .0001$).

Table 5: Fluidity slopes of HPM with phospholipase A_2 from snake venom with or without dBSA.

Concentration of dBSA (mg/mL)	Slope ± S.E. (PV/min x 10^5)	Model R^2
0.0 Control*	17.5 ± 20.9[a]	0.00743
0.0001	-21.4 ± 8.0	0.07032
0.01	-26.2 ± 3.9	0.32137
1.0	-13.7 ± 5.1	0.07107

* All samples contained 0.0001 μg/mL phospholipase A_2 from snake venom.

[a] Concentration of dBSA affected the slope ($p = .0001$).

relative changes over time did not differ due to phospholipase A_2 ($p = .7143$) from bee venom (figures 12-15) or snake venom (figures 16-19).

The initial polarization values of the four samples treated with bee venom phospholipase A_2 were not different (table 6). The initial polarization values for HPM treated with phospholipase A_2 from snake venom and 1 mg/mL dBSA exceeded ($P < 0.05$) samples containing less or no dBSA (table 7).

3.4 EXPERIMENT FOUR

The changes in HPM fluidity monitored over time with t-PnA and c-PnA differed (figures 20, 21, 22 and 23). In the absence of Ca^{2+}, t-PnA detected a decrease in HPM fluidity until 35 ± 17 minutes (table 8), followed by an increase in fluidity. Fluidity of HPM assessed by t-PnA with Ca^{2+} increased linearly. Without Ca^{2+}, the c-PnA detected an initial decrease in HPM fluidity until 38 ± 7 minutes, after which time fluidity did not change. In the presence of Ca^{2+}, c-PnA detected an initial decrease in fluidity until 31 ± 6 minutes and a subsequent increase in fluidity.

The initial unadjusted polarization values obtained with t-PnA were higher ($P < 0.05$) than those obtained with c-PnA

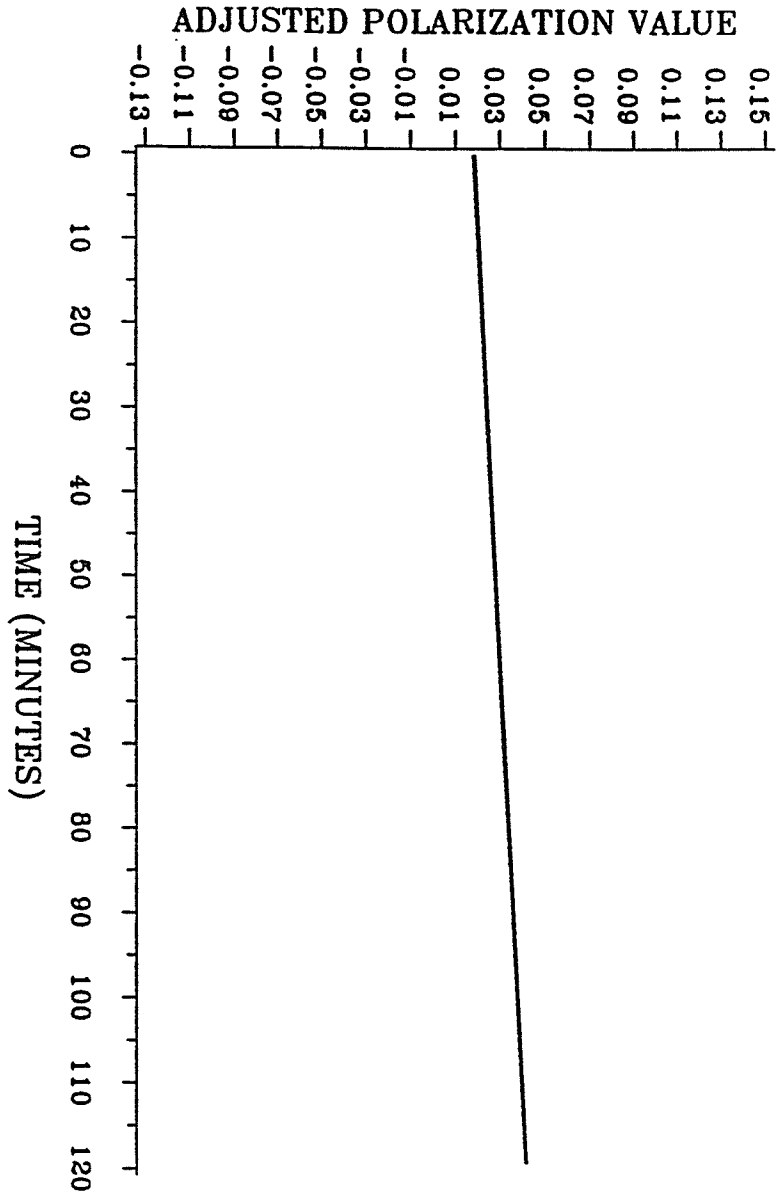
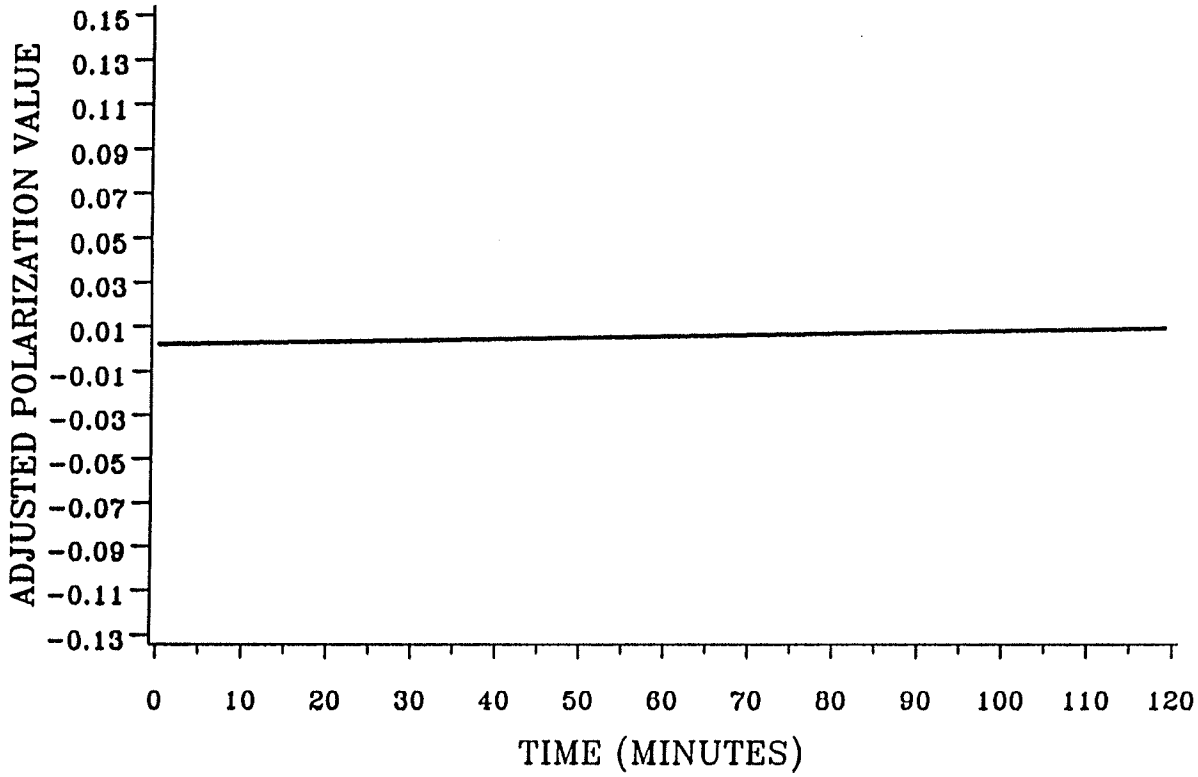


FIGURE 12: FLUIDITY OF HPM WITH BEE VENOM PHOSPHOLIPASE A_2 + 0 mg/ml dBSA OVER TIME

FIGURE 13: FLUIDITY OF HPM WITH BEE VENOM PHOSPHOLIPASE A_2 + 0.0001 mg/ml dBSA OVER TIME



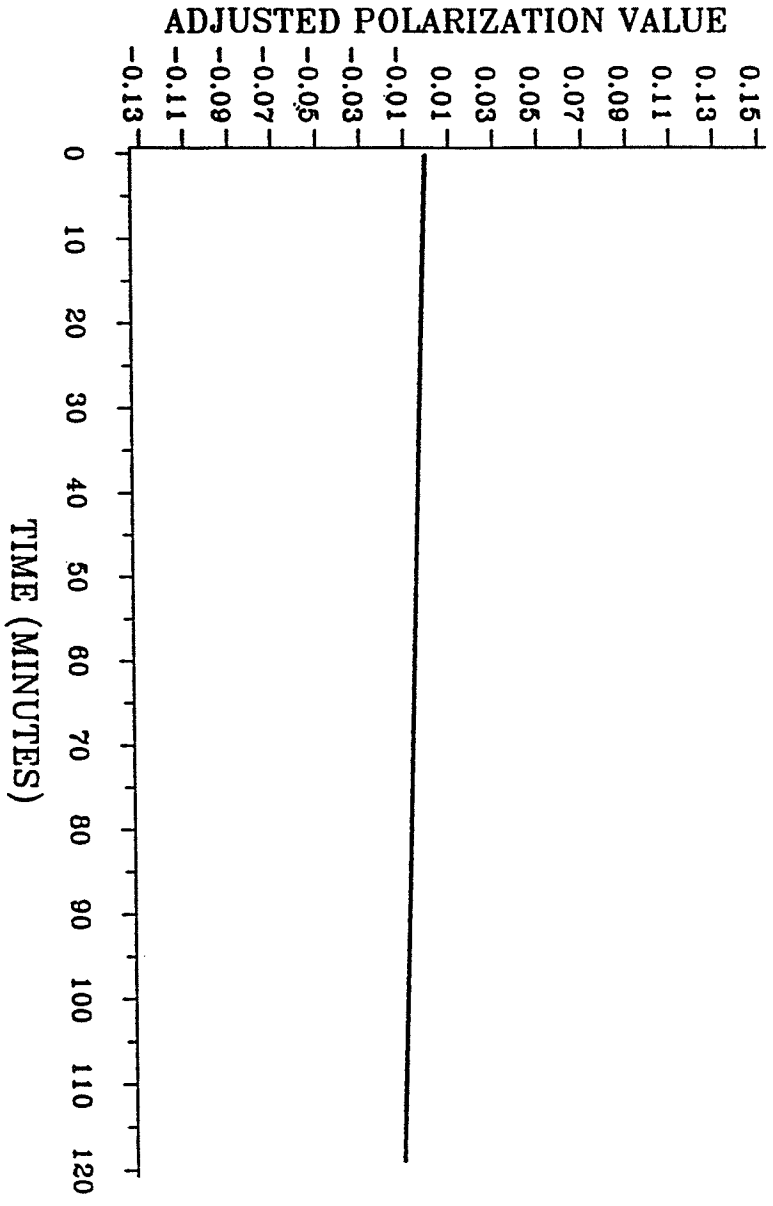


FIGURE 14: FLUIDITY OF HPM WITH BEE VENOM PHOSPHOLIPASE A_2 + 0.01 mg/ml dBSA OVER TIME

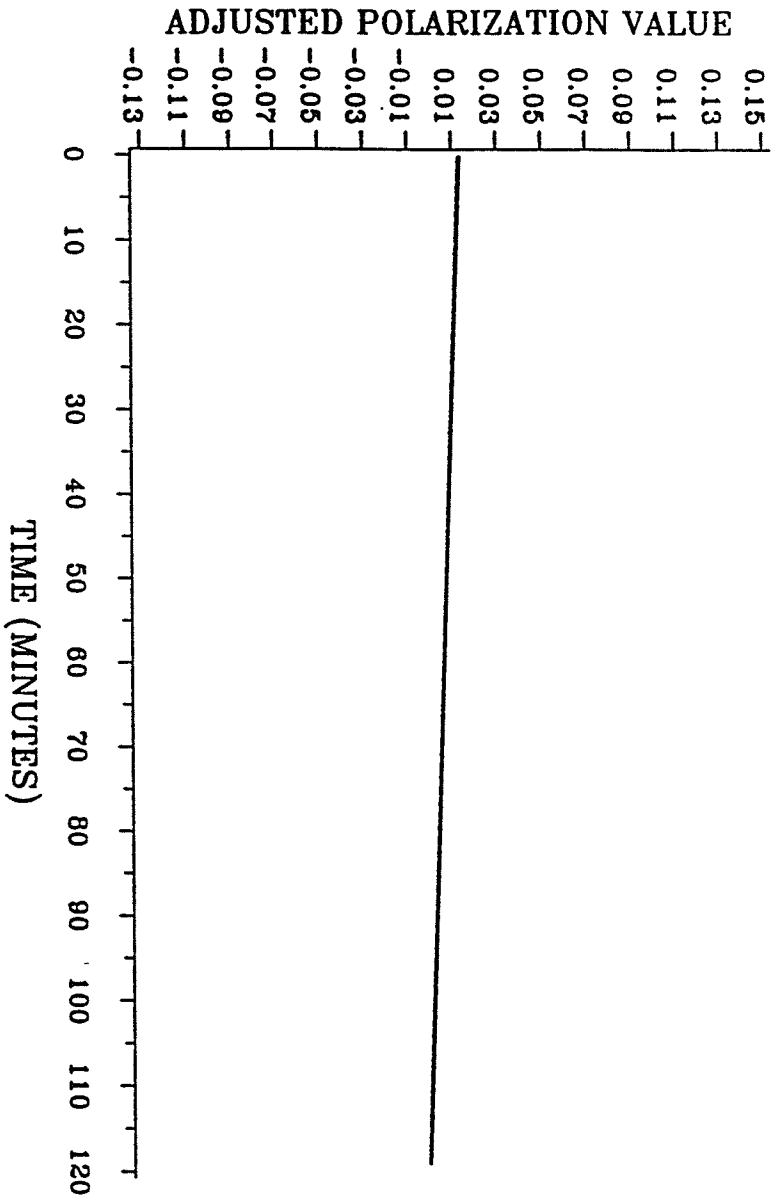
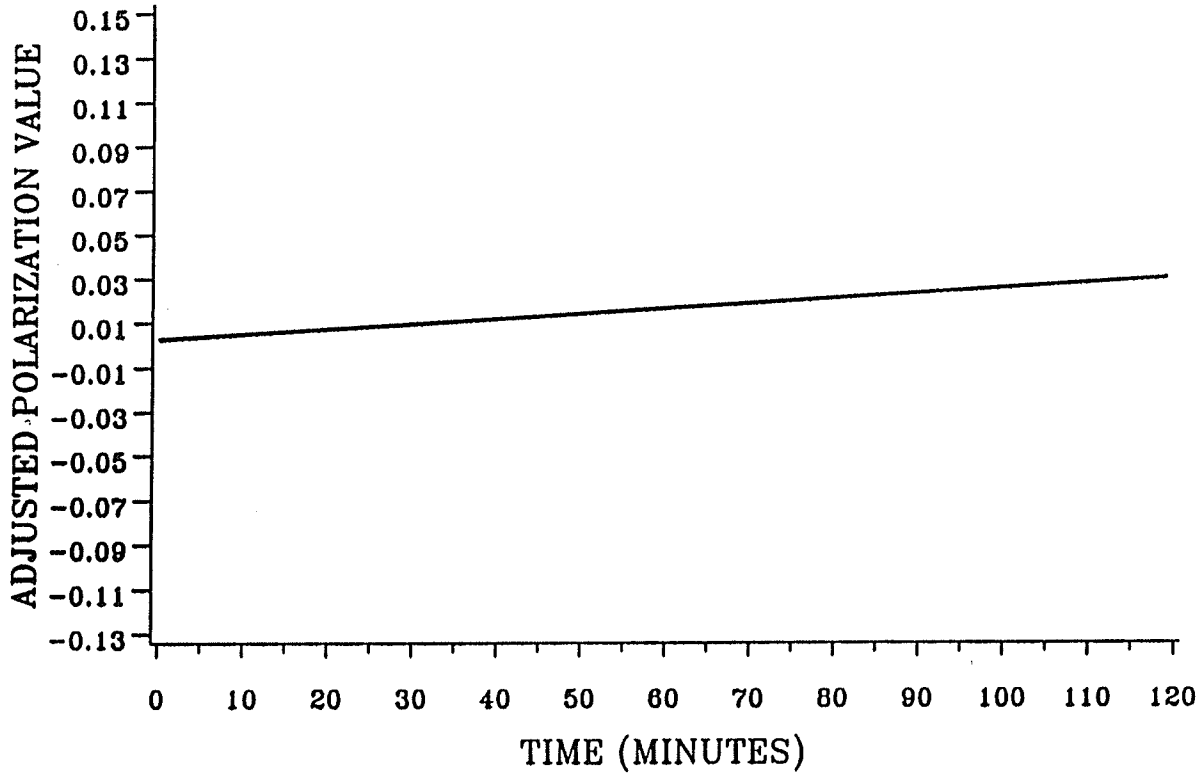


FIGURE 15: FLUIDITY OF HPM WITH BEE VENOM PHOSPHOLIPASE A_2 + 1 mg/ml dBSA OVER TIME

FIGURE 16: FLUIDITY OF HPM WITH SNAKE VENOM PHOSPHOLIPASE A_2 + 0 mg/mL dBSA OVER TIME



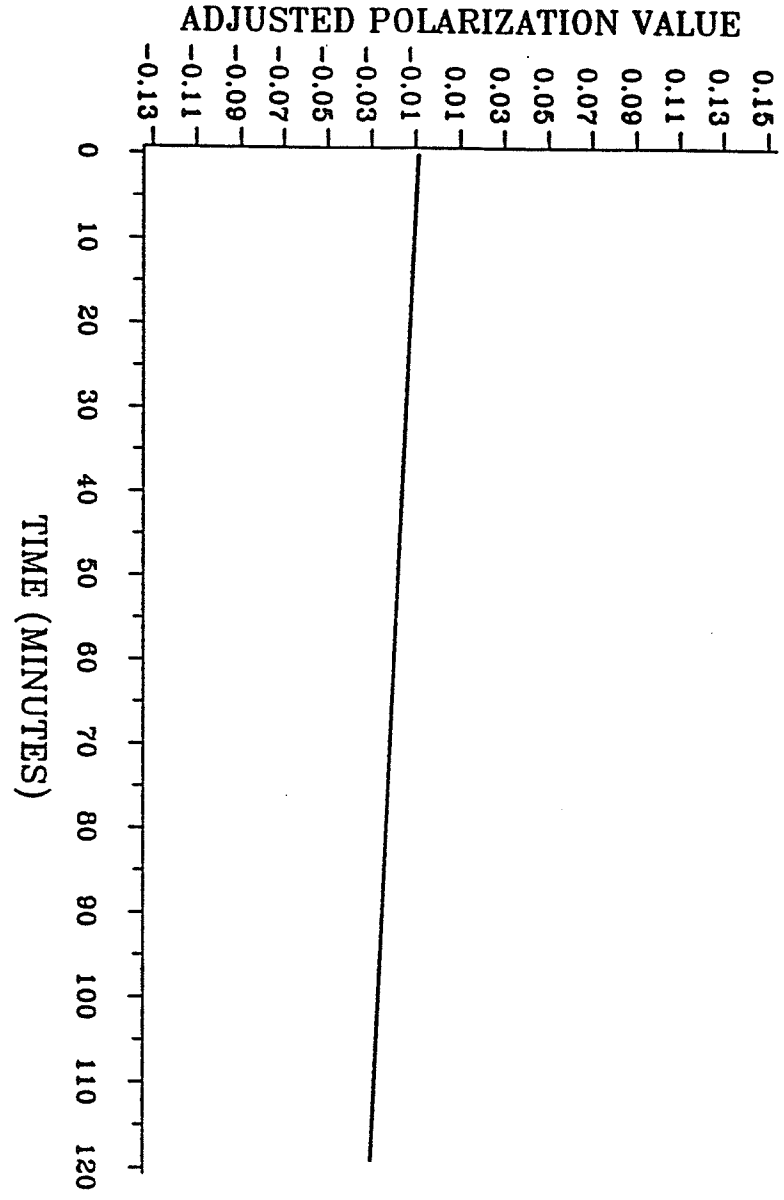


FIGURE 17: FLUIDITY OF HPM WITH SNAKE VENOM PHOSPHOLIPASE A_2 + 0.0001 mg/mL dBSA OVER TIME

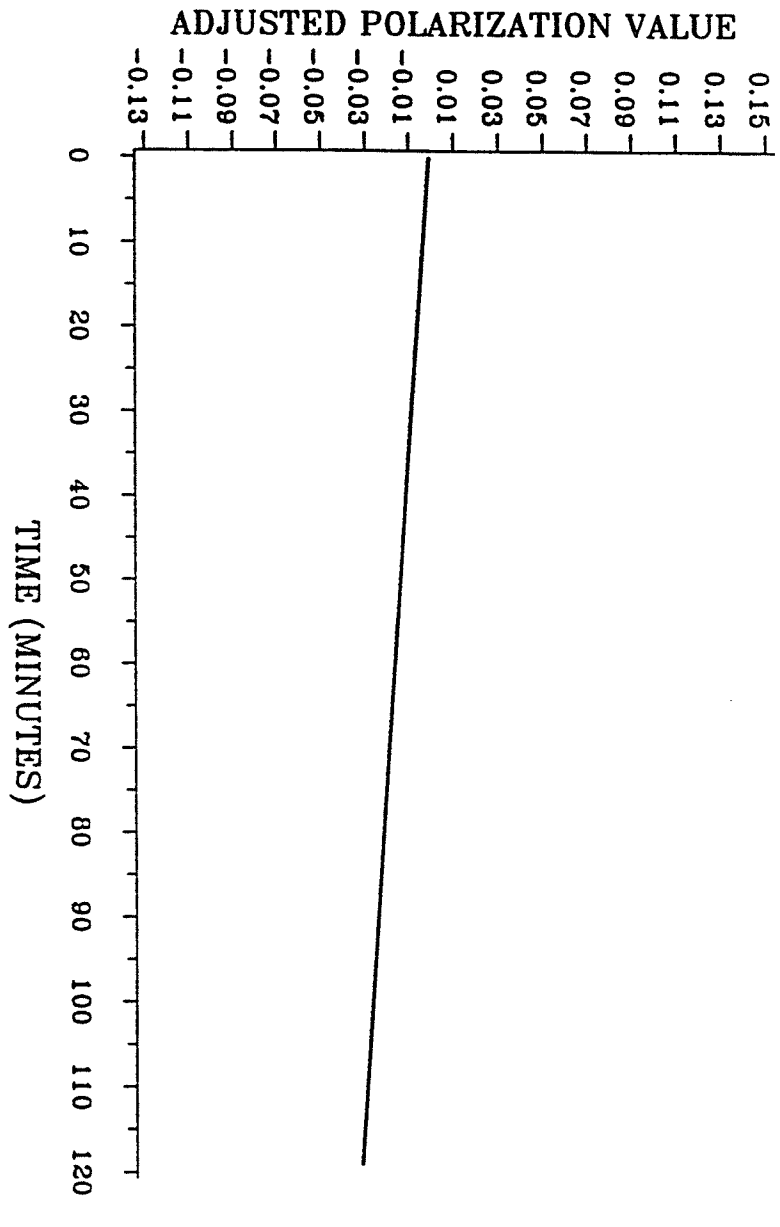


FIGURE 18: FLUIDITY OF HPM WITH SNAKE VENOM PHOSPHOLIPASE A_2 + 0.01 mg/mL dBSA OVER TIME

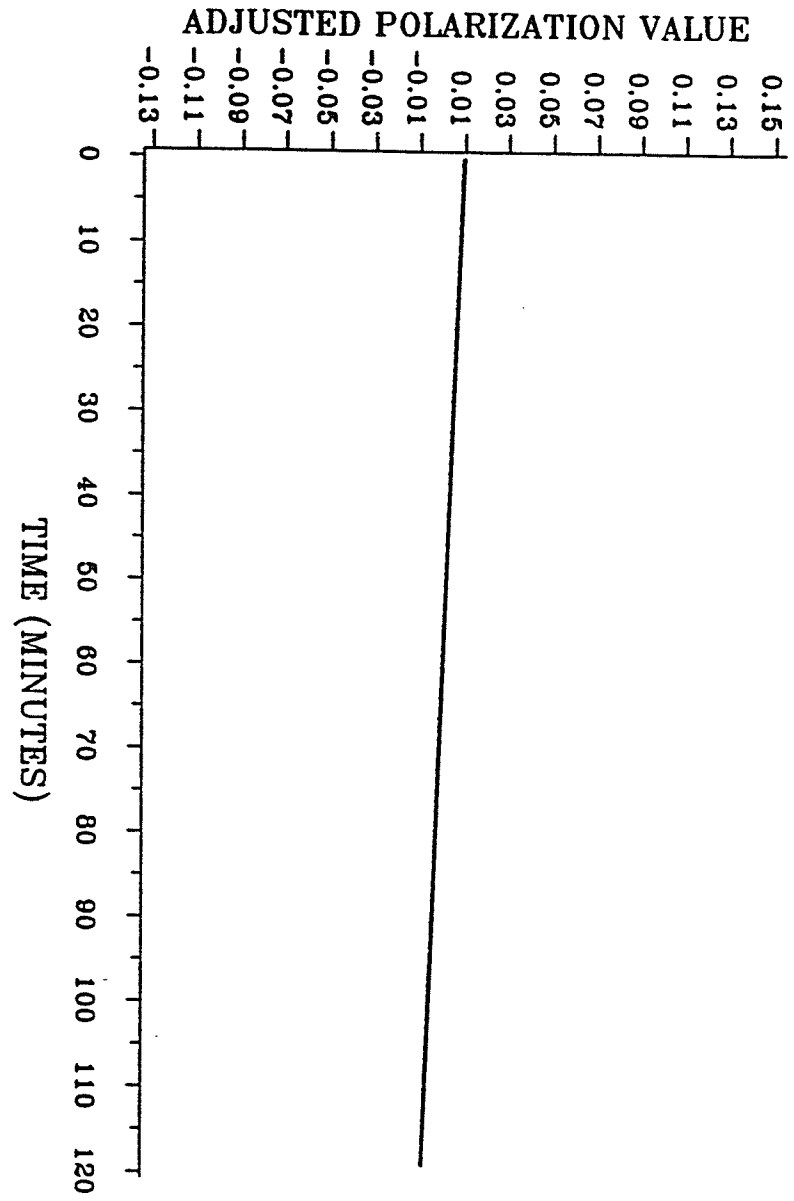


FIGURE 19: FLUIDITY OF HPM WITH SNAKE VENOM PHOSPHOLIPASE A_2 + 1 mg/mL dBSA OVER TIME

Table 6: Initial polarization values (IPV) of HPM containing phospholipase A_2 from bee venom with or without dBSA.

Concentration of dBSA (mg/mL)	IPV (L.S.M. ± S.E.)
0.0 Control*	0.34001 ± 0.03541[a]
0.0001	0.34824 ± 0.02970[a]
0.01	0.37031 ± 0.02631[a]
1.0	0.40590 ± 0.02171[a]

* All samples contained 0.0001 μg/mL phospholipase A_2 from bee venom.

[a] Values with the same superscripts are not significantly different ($p < 0.05$).

Table 7: Effect of dBSA on initial polarization values (IPV) of HPM containing phospholipase A_2 from snake venom.

Concentration of dBSA (mg/mL)	IPV (L.S.M. ± S.E.)
0.0 Control*	0.32983 ± 0.01625[a]
0.0001	0.33371 ± 0.01587[a]
0.01	0.33764 ± 0.02015[a]
1.0	0.40006 ± 0.01795[b]

* All samples contained 0.0001 μg/mL phospholipase A_2 from snake venom.

a,b Different superscripts indicate significant differences ($p < 0.05$).

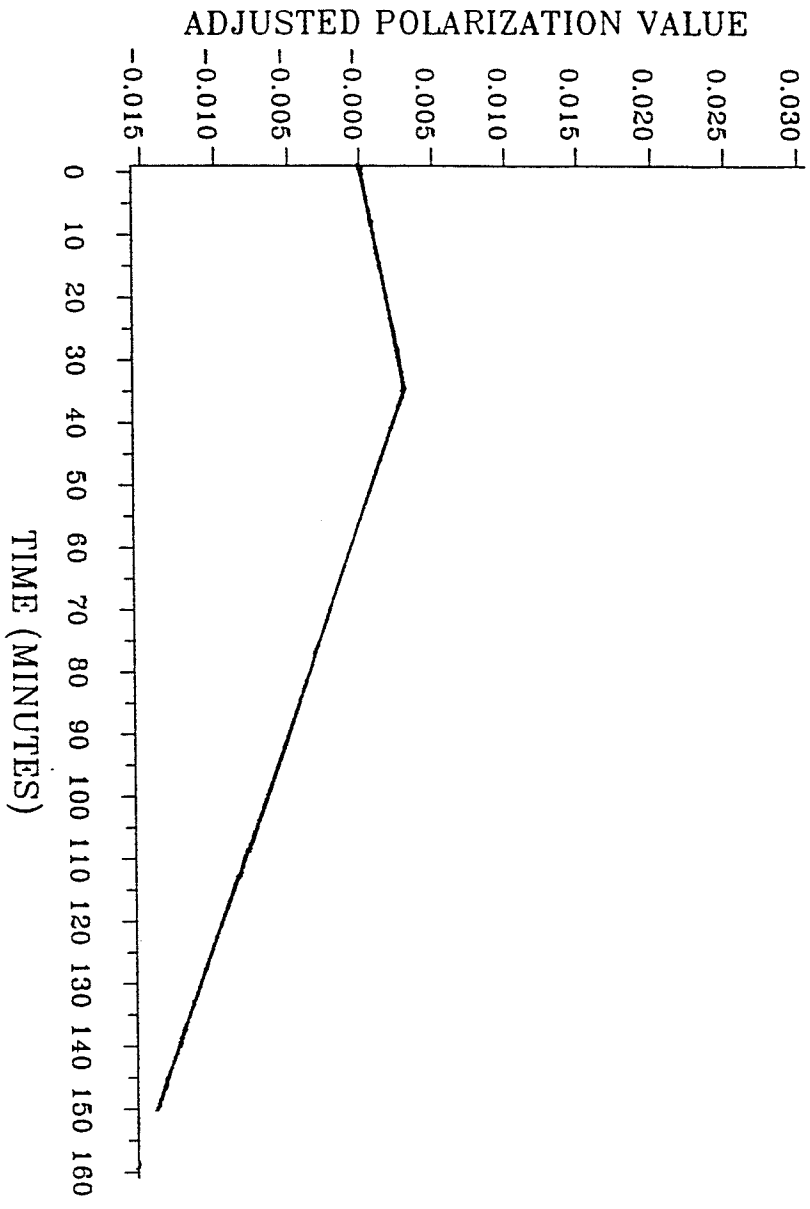


FIGURE 20: t-PNA - DETECTED FLUIDITY OF HPM OVER TIME

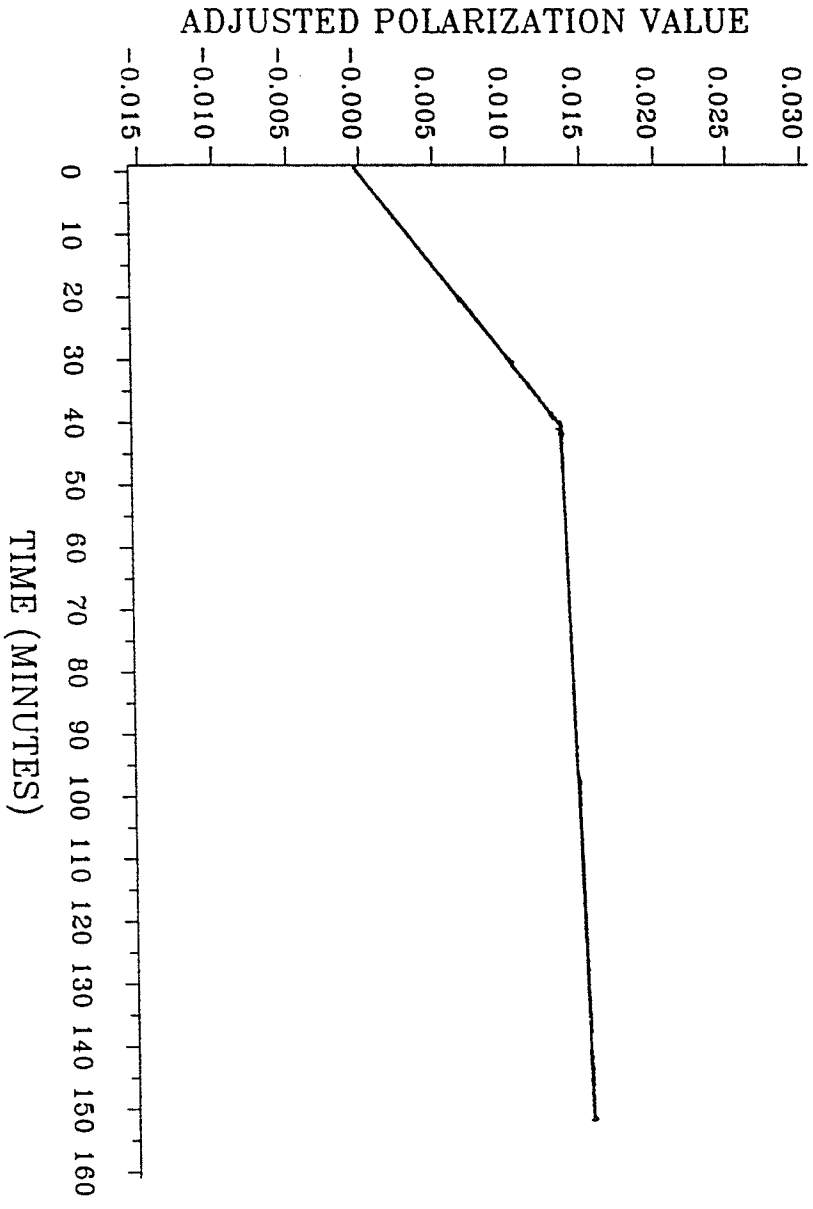


FIGURE 21: C-PNA - DETECTED FLUIDITY OF HPM OVER TIME

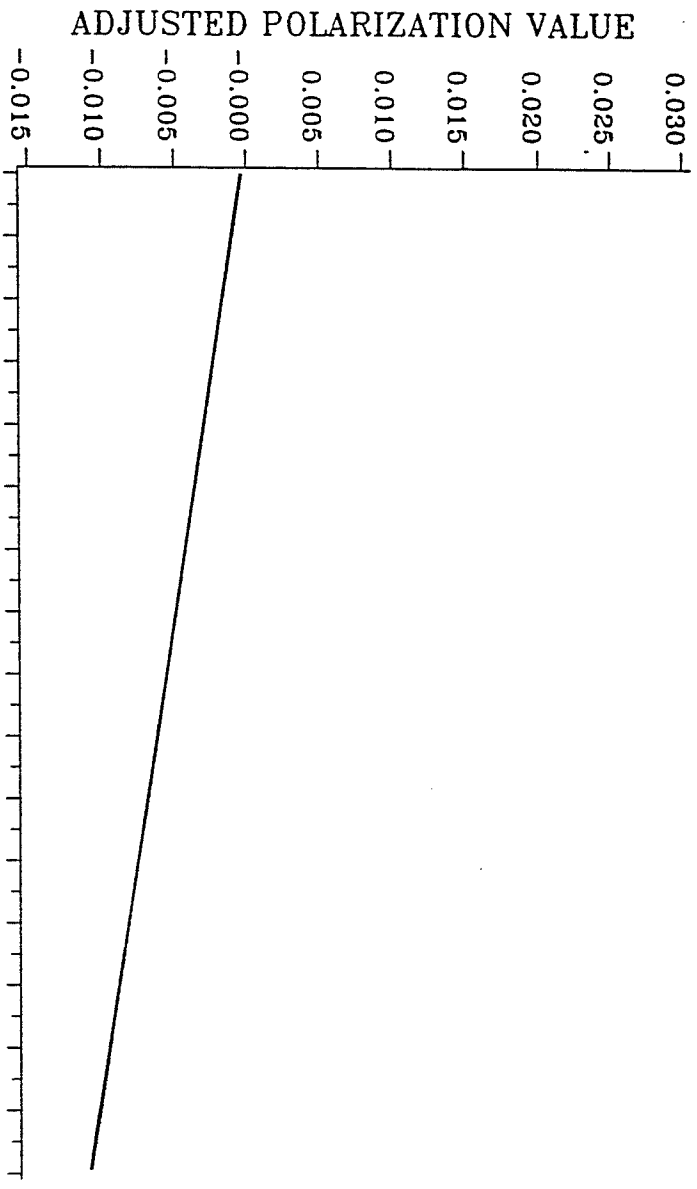


FIGURE 22: t-PNA - DETECTED FLUIDITY OF HPM + Ca^{2+} OVER TIME

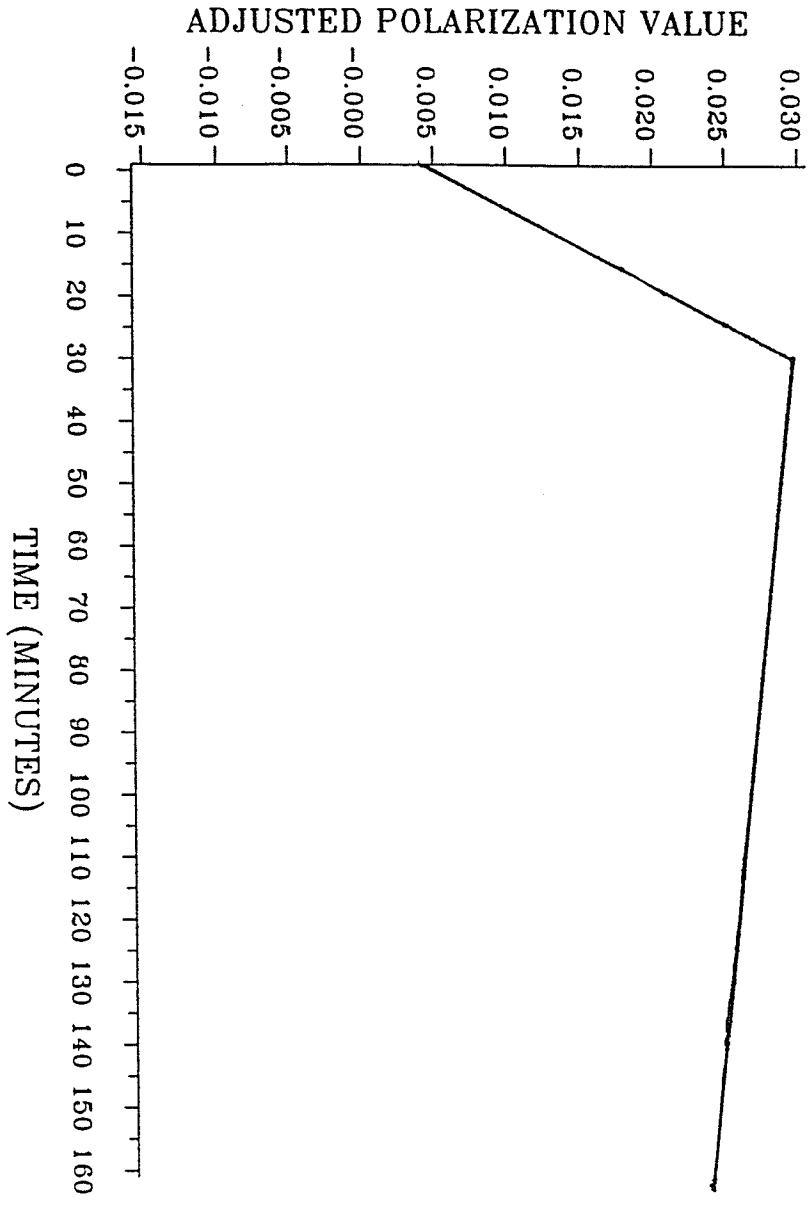


FIGURE 23: c-PNA - DETECTED FLUIDITY OF HPM + Ca^{2+} OVER TIME

Table 8: HPM fluidity changes as detected by trans- and cis-PnA.

Probe	Slope ± S.E. (PV/min x 10^5)	Model R^2
t-PnA slope 1 (0-35 min)	7.5 ± 6.7*	0.11442
slope 2 (35-150 min)	-13.5 ± 2.9	
t-PnA + Ca^{2+}	-10.3 ± 1.4	0.15289
c-PnA slope 1 (0-38 min)	39.5 ± 6.7	0.13206
slope 2 (38-150 min)	1.0 ± 2.3	
c-PnA + Ca^{2+} slope 1 (0-31 min)	85.6 ± 10.3	0.09079
slope 2 (31-150 min)	-5.6 ± 3.4	

* Slopes and breakpoints determined by NLIN procedure (SAS Inc., 1985; see Materials and Methods).

(table 9) and the polarization values detected by t-PnA remained greater than those detected by c-PnA throughout the incubation.

3.5 EXPERIMENT FIVE

The ^{45}Calcium radioassay demonstrated treatment-dependant differences in Ca^{2+} uptake.

Comparisons between treatments within each time interval (table 10a) indicated that treatment 10 (cold shock) promoted a considerably greater Ca^{2+} accumulation by the spermatozoa than the other treatments at all times. Treatments 2 and 3 (1 and 0.01 μg/mL phospholipase A_2 from bee venom) resulted in lower Ca^{2+} accumulations than the other treatments, beginning at 120 minutes. Spermatozoa in treatment 2 accumulated less Ca^{2+} than all other treatments; treatment 3 caused less accumulation than the spermatozoa treated with any dose of phospholipase A_2 from snake venom (treatments 6,7,8,9). The cold shocked spermatozoa immediately underwent massive Ca^{2+} accumulations which were considerably greater than any other treatments. For all treatments, except for the 1 mg/mL bee venom phospholipase A_2 treatment, the spermatozoa accumulated Ca^{2+} over time (Table 10b). Calcium levels began to increase almost immediately at time 0. Overall, treatments containing phospholipase A_2 from bee venom (treatments 2,3,4,5) resulted in less Ca^{2+} accumulation than did the control or treatments

with phospholipase A_2 from snake venom (treatments 1,6,7,8,9).

3.6 ENZYME ANALYSIS

Measurement of the HPM marker enzyme, alkaline phosphatase showed the enrichment of the HPM sample to be 356 ± 52% (mean ± S.E.) as compared to the whole spermatozoa.

Table 9: Initial polarization values (IPV) of HPM analysed by trans- and cis-PnA.

Probe	IPV (L.S.M. ± S.E.)
t-PnA	0.335426 ± 0.002704[a]
t-PnA + Ca^{2+}	0.330437 ± 0.003697[a]
c-PnA	0.272381 ± 0.004712[b]
c-PnA + Ca^{2+}	0.270795 ± 0.005058[b]

a,b Different superscripts indicate significant differences ($p < 0.05$).

Table 10a: Effect of treatment on calcium accumulation by boar spermatozoa (mean ± S.E.M. nM $Ca^{2+}/10^9$ sperm).

TRT	TIME OF INCUBATION (min)								
	0	30	60	90	120	150	180	210	240
1 (control)	6.8± 0.8^{a}	16.3± 3.5^{a}	17.0± 2.5^{ab}	19.0± 4.6^{a}	19.8± 2.1^{a}	21.5± 4.4^{a}	21.6± 1.7^{ad}	20.8± 1.6^{a}	24.6± 6.2^{ac}
2 (1 µg BV/mL)	7.8± 0.6^{a}	10.4± 0.7^{a}	10.1± 1.3^{b}	11.3± 1.9^{a}	11.6± 2.8^{b}	10.9± 1.0^{b}	8.5± 0.4^{b}	8.1± 1.1^{b}	8.9± 0.7^{b}
3 (0.01 µg BV/mL)	8.2± 0.8^{a}	18.4± 3.8^{a}	15.3± 2.7^{ab}	17.5± 5.3^{a}	15.5± 2.5^{ab}	18.8± 4.6^{ab}	16.1± 1.2^{c}	12.9± 2.1^{bc}	15.3± 1.8^{bc}
4 (0.001 µg BV/mL)	8.9± 0.7^{ab}	19.1± 5.5^{a}	20.1± 5.2^{a}	26.1± 5.1^{a}	21.5± 3.1^{a}	23.9± 3.1^{a}	22.3± 2.5^{ad}	19.1± 2.5^{ac}	23.0± 4.0^{ac}
5 (0.0001 µg BV/mL)	7.1± 1.0^{a}	20.0± 3.8^{a}	18.4± 2.3^{ab}	22.3± 5.6^{a}	23.0± 4.9^{a}	18.4± 1.9^{a}	19.1± 2.0^{ad}	18.4± 3.8^{ac}	21.3± 3.8^{ac}
6 (1 µg SV/mL)	9.1± 0.6^{ab}	20.1± 3.2^{a}	21.3± 2.3^{a}	23.1± 5.9^{a}	22.8± 2.5^{a}	23.3± 3.7^{a}	21.4± 2.2^{ad}	19.5± 2.2^{ac}	21.8± 2.9^{ac}
7 (0.01 µg SV/mL)	10.8± 1.0^{b}	12.4± 1.4^{a}	21.4± 3.8^{a}	25.3± 7.1^{a}	21.3± 2.0^{a}	26.1± 4.6^{a}	26.1± 2.8^{a}	21.1± 2.4^{a}	23.0± 1.9^{a}
8 (0.001 µg SV/mL)	7.0± 1.1^{a}	18.2± 2.8^{a}	14.3± 3.9^{ab}	18.7± 4.3^{a}	18.4± 2.0^{ab}	22.8± 2.9^{a}	20.1± 1.1^{cd}	19.6± 3.6^{ac}	23.0± 1.9^{ac}
9 (0.0001 µg SV/mL)	8.8± 0.8^{ab}	17.2± 4.7^{a}	18.4± 3.6^{ab}	19.8± 5.8^{a}	19.5± 2.4^{ab}	25.0± 4.9^{a}	22.3± 1.2^{ad}	17.9± 2.8^{ac}	24.1± 5.2^{ac}
10 (cold shocked)	44.5±19.2^{c}	232.1±56.7^{b}	345.2±82.1^{c}	346.1±114.4^{b}	450.9± 107.6^{c}	502.4±124.8^{c}	498.8±118.4^{e}	512.6±100.4^{d}	542.4±128.2^{d}

a,b,c,d,e Different subscripts within columns indicates significant differences ($P<0.05$).

Table 10b: Time course of calcium accumulation by boar spermatozoa (mean ± S.E.M. nM $Ca^{2+}/10^9$ sperm).

TRT	TIME OF INCUBATION (min)								
	0	30	60	90	120	150	180	210	240
1 (control)	6.8± 0.8^{a}	16.3± 3.5^{ab}	17.0± 2.5^{b}	19.0± 4.6^{b}	19.8± 2.1^{b}	21.5± 4.4^{b}	21.6± 1.7^{b}	20.8± 1.6^{b}	24.6± 6.2^{b}
2 (1 μg BV/mL)	7.8± 0.6^{a}	10.4± 0.7^{a}	10.1± 1.3^{a}	11.3± 1.9^{a}	11.6± 2.8^{a}	10.9± 1.0^{a}	8.5± 0.4^{a}	8.1± 1.1^{a}	8.9± 0.7^{a}
3 (0.01 μg BV/mL)	8.2± 0.8^{a}	18.4± 3.8^{b}	15.3± 2.7^{ab}	17.5± 5.3^{b}	15.5± 2.5^{ab}	18.8± 4.6^{b}	16.1± 1.2^{ab}	12.9± 2.1^{ab}	15.3± 1.8^{ab}
4 (0.001 μg BV/mL)	8.9± 0.7^{a}	19.1± 5.5^{ac}	20.1± 5.2^{ac}	26.1± 5.1^{bc}	21.5± 3.1^{bc}	23.9± 3.1^{bc}	22.3± 2.5^{bc}	19.1± 2.5^{ac}	23.0± 4.0^{bc}
5 (0.0001 μg BV/mL)	7.1± 1.0^{a}	20.0± 3.8^{b}	18.4± 2.3^{b}	22.3± 5.6^{b}	23.0± 4.9^{b}	18.4± 1.9^{b}	19.1± 2.0^{b}	18.4± 3.8^{b}	21.3± 3.8^{b}
6 (1 μg SV/mL)	9.1± 0.6^{a}	20.1± 3.2^{b}	21.3± 2.3^{b}	23.1± 5.9^{b}	22.8± 2.5^{b}	23.3± 3.7^{b}	21.4± 2.2^{c}	19.5± 2.2^{b}	21.8± 2.9^{b}
7 (0.01 μg SV/mL)	10.8± 1.0^{a}	12.4± 1.4^{ab}	21.4± 3.8^{bc}	25.3± 7.1^{c}	21.3± 2.0^{bc}	26.1± 4.6^{c}	26.1± 2.8^{c}	21.1± 2.4^{bc}	23.0± 1.9^{c}
8 (0.001 μg SV/mL)	7.0± 1.1^{a}	18.2± 2.8^{bc}	14.3± 3.9^{ab}	18.7± 4.3^{bc}	18.4± 2.0^{bc}	22.8± 2.9^{c}	20.1± 1.1^{bc}	19.6± 3.6^{bc}	23.0± 1.9^{c}
9 (0.0001 μg SV/mL)	8.8± 0.8^{a}	17.2± 4.7^{ab}	18.4± 3.6^{ab}	19.8± 5.8^{ab}	19.5± 2.4^{ab}	25.0± 4.9^{b}	22.3± 1.2^{b}	17.9± 2.8^{ab}	24.1± 5.2^{b}
10 (cold shocked)	44.5±19.2^{a}	232.1±56.7^{ab}	345.2±82.1^{bc}	346.1±114.4^{bc}	450.9± 107.6^{bc}	502.4±124.8^{bc}	498.8±118.4^{bc}	512.6±100.4^{bc}	542.4±128.2^{c}

a,b,c Different subscripts within rows indicates significant differences ($P<0.05$).

Chapter IV

DISCUSSION

The results of this project clearly indicate cold-induced damage to boar spermatozoa head plasma membranes. Also, this study is the first to suggest a mechanism of damage to boar spermatozoa during freezing. The information obtained is crucial to further investigations attempting to improve current methods of long-term preservation of boar semen.

4.1 HPM CHARACTERISTICS

Membrane fluidity is inversely correlated with polarization values obtained by spectrofluorimetry. Previous work (Buhr et al., 1988) has shown that the HPM from the sperm-rich fraction of boar semen undergoes a sharp decrease in fluidity (increase in polarization values) over time when analyzed with t-PnA. Because t-PnA preferentially partitions in the gel-phase areas of a membrane and c-PnA partitions equally into gel and fluid regions (Sklar et al., 1977; 1980), evaluation of data simultaneously obtained with both probes gave a more complete picture of membrane molecular organization. The higher polarization values obtained with

t-PnA confirmed that these two probes were monitoring different areas of the membrane. There was a difference between the slopes derived from t-PnA analysis of HPM + Ca^{2+} in Experiments Four and Two. This most likely resulted from using different polarizing spectrofluorimeters. The spectrofluorimeter used in Experiment Two had an L-shaped chamber which required opening four times for each polarization value obtained; the SLM spectrofluorimeter used in Experiment Four had a T-format, sealed sample chamber which remained closed fro all readings. Therefore, the samples in Experiment Two were exposed to high levels of room light, which may have affected the polarization values. The differences between the slopes may have also been influenced by the different temperatures used for membrane isolation and fluidity assessment (30C for Experiment Two and 25C for Experiment Four). This does not affect the interpretation regarding domains, however, as identical aliquots of HPM were assessed by t-PnA and c-PnA within each trial of Experiment Four. Initially, the t-PnA partitioned into the more ordered areas and c-PnA into more fluid areas. These domains were changeable with time and the dynamics of change within the domains differed from each other. Saxena et al. (1986) have also identified domains within sperm membranes, using monoclonal antibodies. The more fluid domains monitored by c-PnA initially underwent a strong decrease in fluidity. This

dominated regions, indicating that the more ordered areas underwent fewer changes in fluidity at 25C than did the less ordered areas. Since c-PnA partitions equally into gel and fluid areas (Sklar et al., 1977; 1980), the great difference between the c-PnA and t-PnA data suggested that highly ordered or gel phase areas were, initially, only a small proportion of the HPM. The less ordered areas, monitored by cPnA, underwent a large decrease in fluidity over time. The common breakpoints observed within the data of Experiment Four suggest that a particular group of phospholipid species underwent a phase shift at approximately 35 minutes of incubation. The large standard error associated with the t-PnA assessed HPM (without Ca^{2+}) indicated that the phase change was more gradual, due to a moderating effect of components which are not as prominent in the c-PnA - monitored regions. The presence of Ca^{2+} caused disordering of the t-PnA - situated domains, while increasing the order of the areas monitored by c-PnA. The overall difference in polarization values and patterns of change detected by both probes reinforce the theory of the presence of separate domains within the HPM of boar spermatozoa.

4.2 EFFECTS OF FREEZING AND THAWING

4.2.1 Cooled versus Frozen-Thawed Semen

The slopes of the cooled HPM were greater than the

frozen-thawed, but were much less pronounced than that reported for HPM from semen which was extended for fresh A.I. (Buhr et al., 1988). The ability to decrease fluidity with time appears to be a normal HPM characteristic and both the cooled and frozen-thawed HPM samples were altered in a way that reduced the ability to change. The HPM from neither the cooled nor the frozen-thawed semen responded to the inclusion of a pharmacological level of Ca^{2+}, whereas Ca^{2+} caused the HPM from fresh extended semen to decrease in fluidity (Buhr et al., 1988). Boar spermatozoa are extremely susceptible to cold shock on rapid cooling (Watson, 1981) and the differences between the HPM from fresh extended and cooled semen may indicate that even the slow cooling of the current preservation process is too fast. Also, the toxic effects of glycerol on spermatozoa are well documented (Sanford et al., 1972; Wilmut and Polge, 1974; Murdoch and Jones, 1978; Mahadevan and Trounson, 1984; Jeyendran et al., 1985) and glycerol could have altered the cooled HPM. In addition to the damages of the cooling rate and the extender used, freezing and thawing the semen further reduced the ability of the HPM to change. Freezing and thawing are obvious insults to boar spermatozoa as indicated by low fertility rates (Corteel and Paquignon, 1984; Maynard et al., 1987). Boar spermatozoa damage has been caused by rapid warming from 5C to 30C (Bamba and Cran, 1985). The cooled semen fraction (5C) underwent membrane isolation procedures at 25C and the HPM

may have been affected by this temperature shift. Bamba and Cran (1988) demonstrated intensified warming injury with increasing semen dilution rate, magnitude of temperature change, and terminal temperature, suggesting that the HPM from frozen-thawed semen suffered deleterious effects due to the thawing techniques used.

The SBM is a complex and uncharacterized fraction consisting of acrosomal, body, and flagellar membranes as well as other cellular components. The SBM differed in composition and structure from the HPM as shown by the differences in fluidity patterns and alkaline phosphatase content of these membrane fractions. The SBM from cooled and frozen-thawed semen demonstrated fluidity decreases which were not different from each other, but were less pronounced than the fluidity decrease observed for SBM from fertile, extended semen (Buhr et al., 1988). Also, the preservation processes dramatically increased the SBM sensitivity to Ca^{2+}. Thus, the cooling and extending processes rendered the SBM sensitive to Ca^{2+}, and freezing and/or thawing increased this sensitivity.

The different responses of the HPM and SBM to freezing and thawing and exposure to Ca^{2+} was indicative of the different components and ultrastructure of these membranes. This investigation of the effects of cooling, and freezing and thawing on the HPM and SBM clearly demonstrated the occurrence of ultrastructural shifts in these membranes. These alterations obstructed membrane behaviour due to the cooling

processes and additionally, due to freezing and thawing.

4.2.2 Cold Shock as a Model for Freezing

In Experiment Two, the HPM from cold shocked semen underwent a greater fluidization than that induced by freezing and thawing; the trends, however were similar. Cold shock treatment renders boar spermatozoa completely infertile (Watson, 1981; Watson and Plummer, 1985), and the fluidization patterns observed support the theory that a reduction in HPM fluidity over time is required for fertility. Cold shocked spermatozoa membranes, therefore, can represent an exaggerated model for the extreme effects of freezing and thawing.

4.3 CHARACTERIZING COLD-INDUCED DAMAGE TO SPERMATOZOA

4.3.1 Phospholipase A_2 Model

Phospholipase A_2 is an acyl hydrolase which cleaves membrane phospholipids and releases lysophospholipids and free fatty acids which are known to disrupt membrane structure (Langlais and Roberts, 1985), thereby fluidizing the bilayer. Phospholipase A_2 from bee venom and snake venom at 0.01 and 0.0001 μg per mL caused the HPM to fluidize. At 0.0001 μg phospholipase A_2 per mL, the slopes were the same as the cold shocked HPM, possibly suggesting a similar mechanism of action of these phospholipases and the cold shock treatment on the domains monitored by t-PnA. Data from the $^{45}Ca^{2+}$ radioassay

indicated that high concentrations of bee venom phospholipase A_2 (1 and 0.01 μg per mL) prevented intracellular Ca^{2+} accumulations compared to the control spermatozoa. These data suggested that bee venom phospholipase A_2 can affect HPM fluidity and can maximize the extrusion of Ca^{2+} from intact spermatozoa. ATPase activity has previously been demonstrated to increase as membrane fluidity increases (Silvus and McElhaney, 1982) and stimulation of a Ca^{2+}-ATPase by bee venom phospholipase A_2 in ram spermatozoa membranes has also been reported (Holt and North, 1986). Bee venom phospholipase A_2, therefore, may directly stimulate the Ca^{2+}-ATPase of boar spermatozoa, inhibiting Ca^{2+} influx. This would prevent the acrosome reaction and, ultimately, fertilization.

Snake venom phospholipase A_2 did not prevent intracellular Ca^{2+} accumulation by intact spermatozoa despite fluidizing the HPM in a manner equivalent to bee venom phospholipase A_2. Phospholipase A_2 from bee venom and snake venom sources have different phospholipid substrates (Reed, 1981) and presumably only the action of bee venom phospholipase A_2 on its specific phospholipid substrate affects Ca^{2+}-ATPase. Thus, both sources of phospholipase A_2 would fluidize the HPM, but their substrates or products may have different membrane functions. An alternate hypothesis is that the Ca^{2+}-ATPase is located in the midpiece/tail region of the spermatozoon (Robertson et al., 1988). The substrate specificity of bee venom phospholipase A_2 still enabled it

alone to affect pump function, but, under this hypothesis, Ca^{2+}-ATPase activity is independent of the fluidity of the HPM.

4.3.2 Cold Shock Damage

Cold shock treatment induced several deleterious events in boar spermatozoa. Light microscopy has revealed several gross disruptions of the head membranes of cold shocked, intact boar spermatozoa (Robertson and Watson, 1986). Cold shocked spermatozoa demonstrated tremendous intracellular Ca^{2+} accumulations as compared to control cells. As well, the HPM of cold shocked cells displayed an increase in fluidity with time as detected by t-PnA. The relationship between these events is unknown, however a possible mechanism can be postulated. The cold shock treatment may have induced denaturation of certain HPM proteins and/or trans-membrane linkage proteins connecting the inner plasma membrane and the outer acrosomal membrane. The HPM ultrastructural rearrangement would have resulted in HPM fluidization, and the elimination of the intermembrane links would have allowed the disconnection of the head plasma membranes as observed by Robertson and Watson (1986). Such gross disruption would have resulted in the formation of uncontrolled pores along the membrane, allowing the massive Ca^{2+} influx. If a Ca^{2+}-transport pump is located in the HPM, it would, therefore, be ineffective in removing the intracellular Ca^{2+}, as the HPM has been separated from the cell proper. If, however, the Ca^{2+}-

pump is located along the midpiece or tail sections, it would still be non-functional due to ultrastructural damage in the pump's vicinity caused by cold shock, or the pump could be flooded with the rapid leakage of Ca^{2+} from the sperm head, and unable to remove the excess Ca^{2+}. As mentioned previously, cold shocked spermatozoa are infertile (Watson, 1981; Watson and Plummer, 1985) and showed an increase in HPM fluidity. Commercially frozen-thawed spermatozoa retain some fertilizing capacity (Larsson, 1978; Paquignon, 1983; Reed, 1985; Maynard et al., 1987) and the fluidity of their HPM underwent a fluidity shift intermediate to, and significantly different from that of HPM from either cold shocked or fresh spermatozoa. This again suggests that cold shocked cells may be of use as an extreme model for freezing and thawing. Based upon the cold shocked spermatozoa damage, a hypothesis regarding freeze-thaw damage can be suggested. Freezing and thawing (or the cooling process prior to freezing) result in ultrastructural shifts in the HPM without causing the gross structural damage of cold shock. This ultrastructural shift may inhibit the ability of the HPM to control Ca^{2+} permeability, by reducing the ability of the HPM to change. This could inhibit the acrosome reaction and fertilization even prior to freezing. This is supported by the lack of Ca^{2+} sensitivity demonstrated by the cooled and frozen-thawed HPM as monitored by t-PnA.

4.3.3 Moderating Phospholipase A_2 Damage to the HPM

Defatted BSA has been shown to restore membrane fluidity by selectively removing free fatty acids and lysophosphatides from the bilayer (Riley and Carlson, 1987). It was hypothesized that dBSA would counter the fluidizing effects of phospholipase A_2 on the HPM. Defatted BSA at 1 mg/mL did decrease the initial polarization values for HPM containing snake venom phospholipase A_2 but not bee venom phospholipase A_2. Slopes generated by HPM incubated with phospholipase A_2 and dBSA demonstrated an apparent fluidizing effect of dBSA which was contrary to the hypothesized action. Thus, although dBSA tended to initially rigidify the overall membrane, it appeared to accentuate the fluidizing action of phospholipase A_2. The dBSA may remove free fatty acids and lysophosphatides from the HPM, while leaving diacylglycerol, which is a precursor for arachadonic acid (Nishizuka, 1984). Arachadonic acid has four unsaturated double bonds and has a pronounced fluidizing effect on membrane systems (Gurr and James, 1980). Arachadonic acid has been associated with phospholipase A_2 activity in membrane systems other than HPM (Riley and Carlson, 1987). It appears, therefore, that dBSA decreased the order of the t-PnA-monitored domains, possibly by promoting arachadonic acid production. The mechanism of action of phospholipase A_2 on boar spermatozoa membranes, therefore, is complex and requires agents additional to dBSA to overcome its effects. As well, evaluation of dBSA -

treated HPM with c-PnA and t-PnA would provide a fuller explanation of these ultrastructural events.

Chapter V

CONCLUSIONS

1. The HPM of boar spermatozoa are composed of microdomains of differing fluidities which may reflect differing molecular components. These microdomains respond differently to Ca^{2+} at 25C.
2. Cooling and the freeze-thaw processes inhibit the ability of the HPM to undergo a change in fluidity.
3. The protocol for preparing boar spermatozoa for freezing and/or the warming associated with the membrane isolation procedure may cause ultrastructural alterations of the HPM and SBM.
4. Freezing and thawing result in HBM and SBM ultrastructural damage.
5. The HPM from cold shocked spermatozoa and phospholipase A_2 - treated HPM preparations undergo fluidity shifts similar to that of HPM from frozen-thawed spermatozoa.
6. Cold shock treatment dramatically reduces the ability of intact spermatozoa to regulate intracellular Ca^{2+} levels. Phospholipase A_2 from snake venom did not affect this function and phospholipase A_2 from bee venom increased Ca^{2+} extrusion.

7. The inclusion of dBSA in phospholipase A_2 - treated HPM samples tended to decrease the initial fluidity of the membrane, while allowing the HPM to fluidize over time at 30C. The dBSA, therefore, does not reverse the effects of phospholipase A_2 on t-PnA - monitored areas of the HPM.
8. Further research is required to more clearly identify the location and mechanism of cold-induced injury to boar spermatozoa.

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Appendix 1: Composition of Beltsville F5 Extender (fractions I and II).

Tes-N-Tris (hydroxymethyl) methyl 2 aminoethane sulfonic acid	1.2 g
Tris (hydroxymethyl) aminomethane	0.2 g
Dextrose, anhydrous	3.2 g
Egg yolk	20.0 ml
Orvus ES paste (Procter and Gamble, Cincinnati, OH)	0.33 ml
glycerol[a]	0.02 mL

Dissolve and bring to 100 ml with distilled water, for fractions I and II.

[a] present in fraction II only

Appendix 2: Composition of Beltsville Thawing Solution.

Dextrose, anhydrous	3.7 g
Sodium citrate dihydrate	0.6 g
Sodium bicarbonate	0.125 g
Disodium ethylenediamine tetraacetate	0.125 g
Potassium chloride	0.075 g

Dissolve and bring to 100 ml with distilled water.