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LOW MOLECULAR WEIGHT CATIONIC AND ANIONIC PEPTIDES FROM MACROMOLECULAR AGGREGATES IN BOVINE SEMINAL PLASMA AND THEIR EFFECTS ON BULL SPERM

A THESIS

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NIAZ MOHAMED RASHED AL-SOMAI



THE UNIVERSITY OF WAIKATO

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To my father and my mother

PUBLICATIONS

The work presented in this thesis has resulted in the following publications:

Al-Somai N, Vishwanath R, Shannon P and Molan PC (1991). Isolation of low molecular weight components from dialysate of bovine semen and their effects on motility of bull sperm. *Proceedings of the 23rd Annual Conference of Australian Society for Reproductive Biology*. Sydney, Australia. pp 128.

Al-Somai N, Vishwanath R, Shannon P and Molan PC (1993). Isolation and characterisation of anionic proteins and peptides from bovine seminal plasma and their effects on sperm motility. *Proceedings of the 25th Annual Conference of Australian Society for Reproductive Biology*. Sydney, Australia. pp 13.

Al-Somai N, Vishwanath R, Shannon P and Molan PC (1993). High molecular weight protein aggregates in bovine seminal plasma produce cationic peptides that affect bull sperm motility. *Proceedings of the 25th Annual Conference of Australian Society for Reproductive Biology*. Sydney, Australia. pp 19.

Al-Somai N, Vishwanath R, Shannon P and Molan PC (1994). Low molecular weight components in bovine semen diffusate and their effects on motility of bull sperm. *Reprod. Fertil. Dev.*, **6**: 165-171.

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ABSTRACT

The detrimental effect of bovine seminal plasma on sperm is believed to be the action of its constituent proteins and peptides. The major proteins in seminal plasma are known to be aggregates of peptides of low molecular weight. Various researchers have reported that the anti-sperm activity in seminal plasma is spread over a wide range of molecular weights. High molecular weight cationic material from seminal plasma is believed to be more damaging to sperm than high molecular weight anionic material. The substance or substances responsible for the detrimental effect have not been fully characterised.

The current study was undertaken to examine, more thoroughly, the detrimental effect of bovine seminal plasma on sperm, to isolate and characterise the component(s) of low molecular weight from the aggregate, and to study the interaction between these components with regard to the formation of aggregates and their effect on sperm motility.

In order to achieve this goal, a method had to be developed to isolate the anti-sperm component(s), to give a better dissociation of the component(s) from the macromolecules and to disaggregate the macromolecules to their constituents. Preliminary investigation, using gel permeation chromatography, indicated that low molecular weight fractions from bovine seminal plasma were more detrimental to sperm motility than those of higher molecular weight. By dialysis in the presence of citrate or under acidic conditions followed by gel permeation chromatography under acidic conditions, the low molecular weight components were released from the aggregates and they were assessed for their detrimental effects on sperm. Although they were found to have more detrimental effect than the parent aggregate, they tended to reaggregate to higher molecular weights under neutral conditions.

Methods evaluated as ways of achieving disaggregation were: dialysis in membranes with cut-off of various molecular weights; gel permeation chromatography under dissociating conditions; selective precipitation with isopropanol; immobilisation of the cationic proteins on a cation exchanger, with extensive washing with acetonitrile at pH 3 to dissociate the anionic component(s), then elution with 0.01 mol/l NaOH; separation of the hydrophobic from the hydrophilic component(s) by immobilisation on a hydrophobic interaction column (Phenyl Superose); and reverse phase chromatography on FPLC columns to separate proteins and peptides. Further

sequential use of these methods on FPLC columns for final purification resulted in the isolation of the anionic and cationic peptides in a reasonably homogenous form.

All the fractions obtained using the above methods were assessed for their effects on sperm washed by centrifugation in Ficoll solution to free them from seminal plasma. The washed sperm were suspended in a defined diluent to provide a good test system for examining the effects of seminal plasma and its fraction on sperm.

From attempts to isolate the damaging component from seminal plasma, it appeared that relatively high molecular weight cationic or anionic proteins retained anti-sperm activity while they were in aggregated form, however, the extent of activity depended on the form of aggregation. The isolated cationic peptide was highly basic in nature, highly active against sperm and failed to form an aggregate by itself under neutral pH. The isolated anionic peptide was hydrophobic in nature, inactive on sperm and failed to form an aggregate by itself. A combination of the active cationic and the inactive anionic peptide succeeded, in a certain ratio, in the formation of a large aggregate at neutral pH. This was demonstrated by a reaggregation study with radiolabelled anionic peptide which showed incorporation in aggregates which eluted at the high molecular weight region indicating that the two peptides were required for formation of aggregates. The combination of the two peptides reduced the detrimental effect of the active cationic peptide.

It is hypothesised that the aggregates in seminal plasma are an assembly of the active cationic peptide with the inactive anionic peptide. This aggregation is a way of attenuating the damaging effect while the release of these low molecular weight from the large aggregates accentuates the damaging effects. The well established antibacterial activity in seminal plasma is highest in the form of high molecular weight aggregates. Aggregation in the form of high molecular weight components may serve to protect the male reproductive tract against bacterial invasion and protect the sperm from the toxic effect of the low molecular weight components by being in the form of high molecular weight aggregates.

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CHAPTER ONE
LITERATURE REVIEW AND INTRODUCTION

Artificial insemination for animal breeding purposes is increasingly of commercial importance. At the New Zealand Dairy Board Artificial Breeding Centre, ejaculates from selected bulls are collected and dispatched in either liquid or frozen form. The method used routinely in storing bull semen for a few days involves carefully cooling to 4-5°C after dilution with a suitable protective medium, or by suppressing motility and metabolism by passing nitrogen gas through the diluent and storing it at ambient temperature (Shannon, 1973). For long term storage, semen diluted with a protective medium is frozen at -196°C in liquid nitrogen.

In liquid form, a dose of approximately $2x10^6$ sperm packed in a 0.25 ml straw is dispatched for one insemination (Curson *et al.*, 1991). The semen, in a diluent containing egg yolk, has a useable life of only three or four days. In the frozen state, the sperm can stay viable indefinitely, but because of the injury caused by cooling and freezing (a phenomenon known as "cold shock") combined with the damaging effects of seminal plasma (Collingwood, 1990), a dose of approximately $20x10^6$ sperm packed in a 0.25 or 0.5 ml straw must be used for each insemination (Curson *et al.*, 1991). Artificial insemination with frozen boar semen results in lower fertility and decreased litter size, compared to artificial insemination with fresh semen or semen stored in the liquid state (Johnson *et al.*, 1981). Damaging components of seminal plasma accentuate the effect of cold shock (Collingwood, 1990; Martinus *et al.*, 1991).

Researchers have been looking at method(s) for increasing the survival of sperm in liquid semen and reducing the injurious effect of the freezing process. Finding the factor(s) which contribute to loss of sperm viability will improve the chances of finding a more effective protective medium, and thus reduce the cost and improve the efficiency of artificial breeding.

The volume and constitution of seminal plasma and the concentration of the sperm in semen vary considerably between species (Mann and Lutwak-Mann, 1981). Despite the great physiological significance of seminal plasma as the carrier of sperm to the female reproductive tract, the functions of the various proteins and polypeptides in seminal plasma are still not sufficiently understood. However, a great deal of progress has been made in some areas.

Seminal plasma provides a medium for the motility of sperm, and several of its constituents are known to assist the sperm in fertilising the egg. For example, the "forward motility protein" in bull seminal plasma is responsible for the induction of forward motility in epididymal sperm (Acott and Hoskins, 1978; Acott *et al.*, 1983; Hoskins *et al.*, 1979). This and other components in seminal plasma are necessary for completion of the mission of sperm. Rozin (1960) reported that the motility of human sperm from an oligoasthenospermic patient was stimulated when the sperm were incubated with normal seminal plasma from fertile men. He also reported that the seminal plasma from oligoasthenospermic semen could decrease the motility of sperm from normal semen. Seminal plasma also contains a number of factors (such as the decapacitation factor) that can prevent the fertilising capacity of sperm (Eng and Oliphant, 1978).

Seminal plasma is also known to have detrimental effects on sperm (Shannon, 1965a; Baas *et al.*, 1983). Shannon (1965a), Dott (1974) and many other researchers have reported that bovine seminal plasma depresses the livability of the sperm. There have been reports that seminal plasma in other species also causes damage to sperm (Dott, 1974; Dott *et al.*, 1979). Collingwood (1990) and Martinus *et al.* (1991) confirmed the deleterious effect of seminal plasma on bovine sperm. Martinus *et al.* (1991) showed that seminal plasma enhances the damaging effect of cooling, freezing and reduces the viability of sperm. More recently, a study by Pruitt *et al.* (1993) has shown that the inclusion of seminal plasma during cold storage is detrimental to equine sperm.

Although contact time between the sperm and seminal plasma upon natural mating is shorter than in artificial breeding, the extent of contact of the sperm with seminal plasma and the role of seminal plasma in the female genital tract is uncertain. In artificial insemination it is of practical significance if the damaging factor(s) in seminal plasma and their modes of action are identified. Removal of these factors from semen may reduce damage to sperm and improve the process of artificial breeding. This may be achieved by removal of seminal plasma but on the other hand, lack of the beneficial factors also may cause problems as in some cases infertility is due to poor motility. Thus, understanding the biochemistry of semen is a key answer to at least some of these problems. Defining the molecular characteristics of the damaging factor(s) is an essential step in this direction.

1.1 Storage problems

1.1.1 Temperature and cold shock

During the process of cryopreservation, sperm go through drastic changes and are subjected to a different environment from that in the natural mating process. Sperm experience changes in environmental temperature because the temperature is lowered to 0-5°C, and either maintained for storage without freezing, or lowered further to that of liquid nitrogen (-196°C). The reverse procedure involves a relatively rapid increase in temperature, typically reaching 30-37°C in a few seconds. In deep-freeze storage of semen there has been a major problem in that sperm lose viability during processing (Jones, 1971). This loss of viability has been correlated with structural changes (Nath, 1972).

The causes and consequences of cellular injury during freezing and thawing of living organisms are complex, and the understanding of the mechanisms involved is incomplete. The mechanical stress imposed by the formation of ice crystals was considered the obvious reason for cell rupture. The morphology of the cell membranes, their integrity, and subsequently their physiological functions would be affected.

Temperature changes of this nature characteristically modify cell membrane structure by altering the physical state of membrane lipids (Karnovsky *et al.*, 1982). This change has been recognised as having profound effects upon membrane properties, including their permeability and ability to undergo membrane fusion reactions (Holt and North, 1986). Its significance is unclear; it may be involved in the deleterious changes which accompany the cooling of sperm for storage (Hammerstedt *et al.*, 1978). Freezing and thawing was found to cause considerable ultrastructural changes to the acrosome and mid-pieces of a high proportion of bull sperm (Jones and Stewart, 1979).

The maintenance of plasma membrane integrity must be crucially important for the sperm, as a number of independent aspects of sperm function heavily involve this organelle. The motile apparatus of the flagellum relies upon controlled membrane permeability. Also, the processes of sperm maturation, capacitation, and ultimately the membrane fusion events involved in both the acrosome reaction and gamete fusion require the participation of the plasma membrane. Clearly, therefore, the preservation of sperm by cooling and deep-freezing should ideally cause minimal

damage to the plasma membrane, allowing it to resume its normal functions when required.

1.1.2 Extenders and cryopreservation

To reduce the effects of cooling and freezing on sperm a suitable extender must be added. After Phillips and Lardy (1940) first reported that egg yolk was beneficial for the preservation of sperm, the addition of egg yolk to extenders to protect sperm from cold shock became widespread.

The question of which components of egg yolk can prevent cold shock and freezing damage has been the subject of several investigations (Blackshaw, 1954; Watson, 1976; Graham and Foote, 1987). Investigators have established that the low-density lipoprotein fraction of egg yolk contains the protective components (Foulkes, 1977; Kampschmidt *et al.*, 1953; Pace and Graham, 1974). The effectiveness of lecithin has been compared with that of egg yolk but relatively few results have been published and these are often contradictory (Martin, 1963; Lanz *et al.*, 1965; Gebauer *et al.*, 1970). Recently, Kolossa and Seibert (1990) reported that the extender best suited to maintain swimming activity consisted of the zwitterionic buffer system HEPES-Tris-Citrate supplemented with 0.9% lecithin. The lecithin concentration in cryoprotective diluents affects the recovery rate (Martin, 1963; Gebauer *et al.*, 1970).

It was proposed by Graham and Foote (1987) that liposomes composed of specific phospholipids might be used to alter sperm in a way that would enhance their ability to survive thermal stress. Liposomes containing various preparations of phosphatidyl choline and cholesterol enhanced sperm survival during storage at 5°C, both in terms of motility and acrosomal integrity (Holt and North, 1988). However, their mode of action is not clear. Previous investigations have concluded that phospholipids and lipoproteins act upon sperm plasma membrane to render it more stable. This might be achieved through the formation of loose interactions with membrane or proteins, or through the actual alteration of sperm membrane properties by the modulation of its lipid composition.

The relationship between egg yolk levels and the toxic effect of seminal plasma is of interest. The higher the level of egg yolk, the greater the protective effect against seminal plasma (Shannon and Curson, 1972b). On the other hand, the higher the concentration of egg yolk, the greater the toxic effect of the aromatic amino acid oxidase in semen, this presumably being due to increased substrate level for enzyme activity (Shannon and Curson, 1972b).

Glycerol has been the primary cryoprotectant for freezing human semen since the first successful procedures were reported in 1953 by Bunge and Sherman. It is generally accepted that relatively low concentrations of glycerol may be used in diluents for boar semen in order to obtain satisfactory post-thaw survival (Paquignon and Courot, 1975; Scheid *et al.*, 1980). The levels of glycerol necessary for adequate cryoprotection were reported to be detrimental to fertilisation by boar sperm (Carbo and Einarsson, 1971; Mazur, 1985) and resulted in damage to acrosomal membranes and altered membrane permeability (Watson, 1981). Sperm leave the glycerol in which they were frozen during their transit to the site of fertilisation. Removal of human and boar sperm from glycerol appears to result in osmotic damage to sperm, which may, in turn, reduce the fertile life of the sperm (Jeyendran *et al.*, 1984; Graham *et al.*, 1987).

The hydrogen ion buffers used for storing sperm or for studies of their metabolism have not been completely suitable. Citrate, veronal, and Tris are satisfactory as buffers for semen diluents (Foote, 1970), but none are entirely inert with respect to cellular function. Phosphate tends to precipitate most polyvalent cations and depresses sperm respiration in some species (Murdoch and White, 1968).

The addition of penicillin to diluent medium for sperm has positive effects on sperm motility after thawing. The penicillin component produced not only bactericidal but also anti-oxidative action (Khaznodar *et al.*, 1986). The inclusion of sodium citrate in the cryoprotective extender increased sperm motility immediately after thawing (Kolossa and Seibert, 1990). The biochemical basis of these beneficial effects has not been elucidated. It is, however, noteworthy that sodium citrate, as a chelating agent, is able to prevent peroxide formation in mammalian sperm (Jones and Mann, 1976).

In all living cells that utilise oxygen for aerobic respiration there is potential for damage to the cell by highly reactive oxygen radicals. As a consequence of this, cells that must survive for long periods in an oxygen-containing environment have internal mechanisms to protect themselves from oxidation (Meister, 1983). However, in the unnatural situation in artificial insemination this protection is not sufficient. The presence of an amino acid oxidase in bull semen was originally reported by Tosic and Walton (1950). This enzyme produces hydrogen peroxide and ammonia by the oxidative deamination of the aromatic amino acids (MacMillan *et al.*, 1972). The addition of catalase to diluents to destroy hydrogen peroxide increases the survival of sperm (Shannon and Curson, 1972b; MacMillan *et al.*, 1972; Shannon and Curson,

1982). Oxygen radicals are damaging to the cell, affecting motility due to destructive effects on the force-generating mechanism through oxidation (Lindemann and Gibbons, 1975). Antioxidants such as superoxide dismustase, catalase, dithiotheritol, and reduced glutathione were tested for their ability to prolong the motility of reactivated sperm (MacMillan *et al.*, 1972; Lindemann *et al.*, 1988). These were found to increase the duration of motility in the sperm preparations (Lindemann *et al.*, 1988).

1.2 Diluents and the effects of osmolarity

When semen is processed for artificial insemination, a diluent is usually added to preserve the sperm and to reduce the concentration of sperm to a suitable level. Several components are included in the diluent to improve sperm survival. They affect the osmolarity, pH, and most importantly, the protein constituents in seminal plasma and their function. The diluent may interact directly with the proteins or promote protein-to-protein interaction.

Sperm are extremely sensitive to the ionic composition of their environment. The survival and/or morphology of the sperm of bull (Drevius, 1972) and ram (Quinn and White, 1969) have been shown to be affected by the osmolality of diluents used for semen preservation. If the diluent is hypotonic or hypertonic, then the sperm will suffer and be susceptible to damage. The damage to sperm caused by hypotonic diluents is due to swelling and membrane rupture (Drevius and Eriksson, 1966), particularly with sudden changes in osmolality (Quinn and White, 1969). In hypertonic solutions shrinkage occurs, although ram and rabbit sperm were found not to act as perfect osmometers in contrast to bull sperm (Hammerstedt *et al.*, 1978). Bull sperm swell in hypotonic media and also in media of penetrating non-electrolytes. Under hypotonic conditions sperm from the bovine caudal epididymis rupture at 44 mOsm after a four-fold increase in cell volume (Drevius, 1972).

There is general agreement that hypertonic diluents are less destructive than hypotonic diluents (Mann, 1964), and are superior to isotonic diluents when cells are preserved by freezing (Watson, 1979). It has been demonstrated that ram sperm show better post-thaw motility after being frozen in hypertonic diluents compared with hypotonic diluents (Fiser *et al.*, 1981; Watson and Duncan, 1988). However, a slight reduction in osmolarity is not destructive to sperm, at least in some species. Incubation of equine sperm in normal and low ionic strength media at 37°C, without freezing, showed significant beneficial effects of low ionic strength on motility over eight hours (Bize and Driscoll, 1988). It was found that sperm motility and survival

were considerably enhanced if the osmotic pressure was lowered by 20% from the natural level of 290 mOsm (Bavister, 1974) Progressive motility of bovine sperm during incubation at 5°C or 37°C is maintained longer in 250 mOsm buffer than in solutions of higher or lower osmolalities (Jones and Foote, 1972)..

1.3 Metal ions in seminal plasma

Metal ions are present in seminal plasma. Generally, the concentration of sodium in seminal plasma is significantly higher than that of potassium (Skandhan *et al.*, 1978; Grigis *et al.*, 1980). An optimal intracellular and extracellular concentration of sodium and potassium ions appears necessary for motility and fertility (Skandhan and Mazumdar, 1981). A high ratio of K^+ to Na^+ is considered to be responsible for the immobility of sperm collected from the epididymis (Blean *et al.*, 1979). A reverse pattern in ejaculated sperm has been established (Skandhan *et al.*, 1978). The importance of ions in the enzymatic processes that provide energy for motility is well documented (Mann and Lutwak-Mann, 1981). Shannon and Curson (1987) concluded that extracellular K^+ is an essential component in diluents to maintain viability when sperm are stored at 37°C, and incubation with only Na^+ in the diluent reduced viability.

The function of calcium ions in the process of capacitation is well known. Capacitation is a functional change of sperm during which subcellular changes occur and induce an influx of calcium ions into the sperm, thus triggering the acrosome reaction and hyper-activated motility (Bedford, 1983). The role of Ca^{2+} in sperm motility is controversial as it has been observed that Ca^{2+} may have either an inhibitory or excitory effect. When Davis (1978) examined the effects of Ca^{2+} on the motility and fertilisation rate of rat sperm *in vivo* it was noted that sperm motility was inhibited when the concentration of Ca^{2+} was 1.7 mmol/l, but when it was 3.4 mmol/l, vigorous motility continued for about 2 hours. Fertility was reduced at the low concentration and was higher at the higher concentration.

Seminal plasma contains large quantities of other divalent ions such as Mg^{2+}, which are known to activate the progressive motility of washed sperm (Magnus *et al.*, 1990)

Zinc is also present in seminal plasma. Fini *et al.* (1991) reported that atomic absorption spectrophotometer measurements showed that the zinc content in bull seminal plasma was 2 mg/100 ml, and 8 mg/100 ml in human seminal plasma. Zinc at this concentration may play a role in regulating enzyme functions; for some enzymes, such as the 5'-nucleotidase, it acts as an inhibitor (Fini *et al.*, 1991).

1.4 The effects of dilution on sperm

The addition of extender to semen for the purposes of cryprotection and to reduce the sperm concentration also results in components of seminal plasma being less concentrated than in the native state. One of the most pronounced adverse effects on sperm motility arises simply from excessive dilution of sperm. The sperm shortly after a brief burst of motility become immotile, a phenomenon described for several species (Mann, 1964). Attempts were made to prevent or reduce the effects of dilution on sperm, usually by adding various known chemical substances to the diluent, but these efforts appear to have had limited success because the precise reason for the adverse effect of dilution on sperm motility is unknown. However, from early observations with mammalian sperm, it seems likely that excessive dilution (or washing) of sperm causes loss of cellular components which are required for motility (Emmens and Swyer, 1948; Cheng *et al.*, 1949; Blackshaw, 1953). In ram sperm, washing with Ficoll appeared to exert a negative effect on the sperm plasma membrane by an alteration in the membrane lipid components (Janesen *et al.*, 1982).

Excessive dilution is harmful to ram, bull and rabbit sperm but the viability of these sperm in diluted semen is improved by adding accessory secretion or seminal plasma (Blackshaw, 1953; Emmens and Swyer, 1948), although Shannon (1965b) reported that dilution of seminal plasma had no detrimental effect on the storage properties of bull semen. It was speculated that the detrimental effects of dilution, in rabbit semen at least, were due to dilution of plasma material and loss of substances from sperm (Blackshaw, 1953; Emmens and Swyer, 1948). Hamster sperm are extremely sensitive to dilution in culture medium, which rapidly depressed or even abolished their motility (Bavister, 1974). The dilution effect was diminished when the epididymal hamster sperm were incubated with the extract of washed frozen-thawed hamster sperm (Bavister, 1974). The severe effect of dilution on the motility of sperm is thought to be due, in part, to the loss of potassium and magnesium along with other substances from the cell. The loss of motility is probably due to reduced activity of the Na^+/K^+ Mg^{2+}-dependent ATP-ase (Petersen and Freund, 1976).

MacMillan *et al.* (1972) reported that the dilution effect may be due to peroxide produced from the action of amino acid peroxidase on the aromatic amino acids since the beneficial effect of catalase was most pronounced at low sperm concentration. Shannon and Curson(1972a, b) reported that the addition of dead sperm reduced the life of incubated sperm.

1.5 The sperm membranes

1.5.1 Fragility of sperm

The observation of Cardullo and Cone (1986) that rat sperm are more susceptible to damage than bull sperm when the fluid in which they are suspended is stirred, raised the question of whether the greater fragility of rat sperm was due to their greater length. If larger sperm are indeed more fragile, there would be many mammalian species with fragile sperm. The lethal shear stress for bull sperm is 4200 dynes/cm^2 (Baltz *et al.*, 1990). Mammalian sperm are subjected to potentially lethal levels of shear stress *in vivo*, therefore strengthening of the sperm by large dense fibres appears to be necessary to insure their survival. In addition to these dense fibres, large mammalian sperm may be protected from damage by other mechanisms (Baltz *et al.*, 1990).

1.5.2 Membranes and surface charges

The outer surface of the sperm has been the subject of intensive investigation during the past few years. These studies have clearly indicated that the composition of the mammalian sperm surface undergoes molecular changes during epididymal transit and capacitation and that these changes are necessary for the sperm to achieve fertilising ability. Characterisation of the surface proteins of ejaculated bull sperm revealed 6 fractions ranging in molecular weight between 14 and 67 kDa (Vierula and Rajaniemi, 1980). Vierula and Rajaniemi (1980) reported that electrophoresis of labelled seminal plasma displayed two fractions which coincided with two fractions of the sperm. This observation suggests that the surface of ejaculated bull sperm and seminal plasma may have some protein components in common which further supports the fact that there may be an interaction between the surface of the sperm and the surrounding seminal plasma proteins (Voglmayr *et al.*, 1979). Vierula and Rajaniemi (1980, 1981) suggested that a loosely bound 17 kDa protein, also present in seminal plasma, is adsorbed by the sperm surface from seminal plasma and possibly originates from the accessory glands. Vierula and Rajaniemi (1983) reported that the increase in the intensity of the 17 kDa protein peak on the surface of the sperm during incubation of unlabelled sperm with radioactive labelled seminal plasma suggests that only the 17 kDa protein is continuously displaced by proteins from seminal plasma.

The orientations of sperm in an electric field indicates that the head and tail have different surface charges (Bedford, 1963). The tail plasma membrane of ejaculated sperm usually has a higher binding capacity for colloidal iron hydroxide than the head

plasma membrane suggesting that the tail has a higher net negative charge than the head (Cooper and Bedford, 1971).

Other studies have also demonstrated regional differences in surface properties of the mammalian sperm (Cooper and Bedford, 1971; Vierula and Rajaniemi, 1981). These studies indicate that the regional differentiation of the surface components of sperm has significant roles in the fertilisation process but the macromolecular nature of these components has remained unknown. Components of the extracellular matrix (including proteoglycans) influence adhesion, motility, proliferation, growth and differentiation of many cells (Wicha *et al.*, 1982; Hook *et al.*, 1984, Ratner *et al.*, 1985; Blum *et al.*, 1987). Extracellular matrix proteoglycans, composed of a protein core to which glycosaminoglycan chains are attached, are also involved in fertilisation in a number of species. A heparin-like glycosaminoglycan from the bovine oviduct has been implicated in capacitation of bull sperm (Parrish *et al.*, 1988, 1989). Miller *et al.* (1990) found that bovine sperm that have been exposed to seminal plasma possess more binding sites for heparin than sperm from the cauda epididymis that have not been exposed to secretions of the accessory sex gland. Recently, Ravnik *et al.* (1992) reported the purification of a lipid transfer protein from human follicular fluid. It was proposed that the mechanism of action of this protein is similar to that of LTP-I, a protein in human plasma which facilities exchange and net transfer of lipid among the plasma lipoproteins (Albers *et al.*, 1984; Tollefson *et al.*, 1988). Changes to sperm membrane lipids during capacitation, particularly the loss of cholesterol, were proposed to be due to the presence of specific lipid transfer proteins which are present in the reproductive fluids (Ravnik *et al.*, 1992). Loss of cholesterol could affect the membrane fluidity, leading to a fusion-competent membrane in preparation for the acrosome reaction (Langlais and Roberts, 1985).

1.6 Constituents in seminal plasma and their effect on sperm

The biochemical constituents of seminal plasma play an important role in preventing effective cryopreservation. A high protein content in seminal plasma lowers the ability of bull sperm to withstand freezing (Pangawkar *et al.*, 1988). This may be due to the high protein content causing an increase in the permeability of the sperm membrane, thus promoting cold shock and injury to the cells during freezing (Hibbitt and Benians, 1971). A high protein content has also been reported to render the boar sperm membrane sensitive to the noxious effect of cooling and deep freezing (Lavon and Boursnell, 1971).

Components in seminal plasma were implicated in enhancing the damaging process. Seminal plasma increases the detrimental effect of cold shock on boar sperm (Pursel *et al.*, 1972). It is possible that exposure of sperm to seminal plasma at 5°C for an 18 hour period prior to freezing results in destabilisation of the sperm membrane, rendering it more susceptible to freezing damage and/or degradation prior to freezing. Moore and Hibbitt (1976) postulated that the external membrane of bull sperm serves as a binding site for proteins originating from seminal plasma which adhere and affect it adversely during cooling, consequently lowering the resistance of sperm to deep freezing.

Seminal plasma, the fluid which serves as a vehicle for ejaculated sperm, is a mixture of secretions of the testes and the accessory glands (Mann, 1975). The bulk of seminal fluid, the seminal plasma, is provided by the male accessory organs: epididymis, vas deferens, ampulla, prostate, seminal vesicle, Cowper's gland, coagulation glands and Littre's gland. Bull seminal plasma has been most extensively studied, but other seminal plasma like that from stallion (Szumowski and Stober, 1955) and boar (Boursnell *et al.*, 1962) has also been studied.

Seminal plasma of all mammals is rich in proteins. The protein concentration of whole seminal plasma of four species before dialysis was reported by Bennett (1965) to be: bull 62 mg/ml, rabbit 22 mg/ml, ram 40 mg/ml and boar 35 mg/ml. Other reports vary considerably. A report from Moustafa and Meszarose (1980) indicates that the concentration of protein in bull seminal plasma is between 58 and 80 mg/ml, while Pangawkar *et al.* (1988) reported it to be between 61 and 67 mg/ml and Patel *et al.* (1988) reported 102.9 ± 0.13 mg/ml. Manjunath and Sairam (1987) recovered about 200 mg of protein from 3 ml of bovine seminal plasma. The total amount of protein in human seminal plasma is impressive if one compares it with other body fluids: between 30 and 65 mg/ml has been reported (Lizana and Eneroth, 1985).

During passage through the epididymis sperm acquire the potential for progressive movement and ability to fertilise ova (Hamilton, 1977). Sperm interact with specific proteins from the luminal fluids of the epididymis (Voglmayr *et al.*, 1980 and 1982) and it is possible that such proteins may be concerned with the maturation process.

Several studies have demonstrated the presence of epididymal proteins on the sperm membrane which are of importance for the function of sperm. For example, the epididymis-specific proteins bound to sperm may play a role in preventing the occurrence of premature acrosome reaction in the male genital tract by acting as

antagonists of capacitation (Kohane *et al.*, 1980). A recent study by Vreeburg *et al.* (1992) showed that after retrograde perfusion of the superior and inferior epididymis with labelled methionine, about 90% of the amount of labelled methionine was taken up during the first passage of sperm through the organ. Vreeburg *et al.* (1992) concluded that some epididymal proteins bound firmly to sperm. Such an event may involve remodelling of the plasma membrane which is thought to be of critical importance since the sperm surface is involved in inter- and intracellular aspects of the fertilisation process (Eddy, 1988). Of the various proteins attached to the sperm in the caput and the corpus epididymides only a few proteins remained associated after washing with high salt buffer (Vreeburg *et al.*, 1992). It has been reported that antibodies to these proteins inhibit the fertilising ability of the sperm (Cuasnicu *et al.*, 1984).

Rat testicular sperm acquire the ability to be motile during epididymal maturation but are stored in an immobile state in the cauda epididymis due to the presence of a glycoprotein with a molecular weight of 200 kDa which is responsible for the high viscoelastic drag of the cauda epididymal fluid. Decreasing the drag increases the motility of sperm, and sperm exhibit active mobility only on ejaculation (Usselman and Cone, 1983).

White *et al.* (1978) confirmed the result obtained by Voglmayr and White (1979) that cauda epididymal fluid greatly increases the oxygen uptake and motility of washed ejaculated ram sperm. Cauda epididymal fluid contains low molecular weight substrates, which serve as oxidisable substrate for sperm (Jones, 1978), as well as a high molecular weight fraction (>5 kDa protein), which is absorbed, internalised and processed by sperm (White *et al.*, 1978; Voglmayr *et al.*, 1982).

Heyns (1977) isolated a steroid-binding protein from the prostatic cytosol of rat, the prostatic binding protein. Prostatic binding protein is probably secreted by the prostate since the prostatic fluid was found to bind as much steroid as the prostatic cytosol (Heyns, 1977).

The roles of most proteins secreted by the accessory sex gland in the functions of sperm are not sufficiently understood. Although the roles of some of the proteins found in seminal plasma have been described, the roles of most of them remains elusive. However, for some proteins it is well established. The physiological function of one or more seminal vesicle proteins in rodents in the formation of the seminal plug or with coagulation immediately after ejaculation is well known (Williams-Ashman,

1984). Eliasson and Lindholmer (1973) suggested that a beneficial factor is present in the prostatic secretion and a deleterious one in the seminal vesicle secretion.

Salisbury and Cargle (1956) reported the beneficial effect of seminal plasma on sperm come partly from its dilution and stimulatory effects and possibly partly by the beneficial action of specific substances contained in it. It was suggested that there is a non-specific relationship between sperm numbers and seminal plasma proteins in general, rather than a specific relationship with any one protein and that some product of sperm controls the rate of entry of blood plasma proteins into the seminal plasma (Chard *et al.*, 1991).

Boar and ram seminal plasma contain an anti-agglutination protein (Dacheux *et al.*, 1983). The addition of ram cauda epididymal fluid or ejaculated plasma, prior to or after agglutination, prevented or reversed agglutination of sperm.

The influence of different fractions from seminal plasma on sperm viability is controversial. Seminal plasma has been reported to be important for the motility and function of sperm and promotes the survival of sperm in man (Lindholmer, 1974) and bull (Acott and Hoskins, 1978). The addition of fresh seminal plasma to stored semen improves motility, reduces pH and increases the number of motile bull sperm. In contrast, Flips (1954) found that the presence of seminal plasma causes an apparent reduction in the uptake of ^{14}C glucose by sperm cells.

Enzymes in seminal plasma are necessary for completion of the physiological functions of sperm. Seminal plasma contains enzymes involved in nucleotide and nucleic acid metabolism (Mann, 1964). Fini *et al.* (1985) purified 5'-nucleotidase from bull seminal plasma and from human seminal plasma (Fini *et al.*, 1991). This enzyme's role in dephosphorylation of AMP has already been established, and it has been shown that it has a relevant function in the rat heart (Truong *et al.*, 1988). However, it is still not known if such a role could be attributed to adenosine in fertility (Fini *et al.*, 1991). Sialyl transferase is an enzyme involved in the transfer of sialic acid to glycoproteins and glycolipids in the course of their biosynthesis. Sialic acid is claimed to be involved in a variety of biological processes including maturation, function and structural integrity of sperm (Base and Prasad, 1975; Levinsky *et al.*, 1983).

Human seminal plasma contains a number of serine proteases which originate principally from the prostatic secretion (Zaneveld *et al.*, 1975). These serine proteases

are known to be key enzymes that regulate many biological processes through limited proteolysis of inactive precursors. Gel permeation chromatography of bull seminal plasma on Sephadex G-50 yielded three fractions with antitryptic activity (Cechová *et al.*, 1979a). Perhaps these inhibitors protect the sperm from proteolytic activity since sperm get coated with the inhibitors in the epididymis and during ejaculation (Veselsky and Cechová, 1980). Highly purified trypsin-acrosin inhibitors isolated from seminal plasma and the seminal vesicle also inhibited fertilisation in an *in vivo* assay (Zaneveld *et al.*, 1971).

The secretions of the male genital tract of guinea pig, rabbit, boar, bull, horse and man also contains inhibitors of acrosin and trypsin. These inhibitors exhibit anti-fertility activity and their function in the male appears to be to protect the reproductive tract against acrosin or proteases (Fritz *et al.*, 1975). In human seminal plasma, their only well documented role is the hydrolysis of seminal plasma proteins forming the seminal clot (Lilja, 1985). Bull seminal plasma contains acetylcholinesterase. Ibrahim and Gaal (1979) reported that a negative correlation was observed between sperm motility and seminal plasma acetylcholinesterase activity and that a very high level of enzyme activity was noticed in seminal plasma of bulls with poor semen quality. The semen with this high enzyme activity lost some of its motility before and after deep-freezing and could not be used for insemination. The motility and fertility of the sperm were reported to be better before and after thawing if the seminal plasma had a low acetylcholinesterase activity.

Despite the significant contribution of proteins in seminal plasma to the physiological function of semen, its detrimental effect is well documented. Apart from the anti-fertility and decapacitation effects, some factors in seminal plasma are reported to inhibit motility and cause damage to the biological membranes. Factors responsible for these detrimental effects vary in molecular weight and charge.

It has been reported that many proteins of the epididymis and accessory glands bind to sperm and prevent them from undergoing capacitation and acrosome reactions (Yanagimachi, 1988). Chang (1957) reported a detrimental effect of seminal plasma on the fertilising capacity of sperm. Bedford and Chang (1962) showed that detrimental material (termed decapacitation factor) sedimented with ultracentrifugation of seminal plasma. The decapacitation factor is described as a substance that functionally reverses the capacity of sperm to fertilise ova. Eng and Oliphant (1978) reported the isolation from rabbit seminal plasma of a highly purified glycoprotein which reversibly decapacitated rabbit sperm by membrane stabilisation.

It was reported that the acidic glycoproteins in the rabbit cauda epididymal influence the maturation of sperm (Orgebin-Crist and Jahad, 1979), or act as a decapacitation factor (Eng and Oliphant, 1978).

The presence of a high molecular weight anti-fertility factor in human seminal plasma was established by Reddy *et al.* (1979). Audhya *et al.* (1987) reported the purification and partial chemical characterisation of a glycoprotein (AF-1) with anti-fertility activity from human seminal plasma. Anti-fertility proteins are also present in bull seminal vesicle fluid. Chang (1957) demonstrated that the fertilising ability of capacitated rabbit sperm was inhibited by mixing these sperm with bull seminal plasma. Seminal plasma from bull, boar, stallion and primates will also decapacitate rabbit sperm (Dukelow *et al.*, 1967).

Because of the possibility that antibodies may interfere with the physiological function of the sperm, the seminal plasma has special significance due to the presence of some components that possess an antigenic property which acts specifically to provoke or prevent some event that may interrupt the natural process of the sperm. Human seminal plasma contains components capable of suppressing various immune reactions and immunocomponent cells (Alexander and Anderson, 1987). Component(s) of seminal plasma play a pivotal role in the immunomodulation of the female and male genital tracts (Alexander and Anderson, 1987). The term "sperm-coating antigens" has been used to describe the phenomenon of coating the sperm with proteins originating in seminal plasma as they pass along the male reproductive tract (Weil, 1965). It was suggested that components secreted from the epididymis or seminal vesicle bind to ejaculated sperm as sperm coating antigens. These antigens may correspond with some antibodies in the serum of men producing sperm since the correlation between sperm-immobilising antibodies and sterility in humans has been well established (Jones *et al.*, 1973), and antisperm antibodies in infertile patients have been detected (Shulman, 1986). Kamada *et al.* (1991) demonstrated that human seminal plasma contains two novel soluble proteins with estimated molecular weights of 16 and 20 kDa which are capable of binding immunoglobulin G. In 1992 Kameda *et al.* reported that these antigens are present in the human sperm membrane as well as seminal plasma.

Seminal plasma that had been separated from semen by centrifugation showed similar sperm-damaging properties during cryopreservation to those of seminal plasma from vasectomised bulls, *i.e.* plasma that had not previously been exposed to sperm

(Collingwood, 1990). This indicates that prior exposure of the plasma to sperm does not noticeably reduce its ability to cause damage during freezing. The damage to ejaculated sperm caused by freezing can be reduced by removing the seminal plasma, indicating the possibility of a reversible association between damaging seminal plasma constituents and sperm. Seminal plasma is particularly damaging during incubation at 5°C for up to 1 hour: subsequent warming in the presence of seminal plasma increases the degree of cell damage (Collingwood, 1990).

The toxic effect exhibited by seminal plasma from centrifuged whole semen observed early by Shannon (1965a) could have been due to a combination of two factors: one was derived from seminal plasma, and the other, an aromatic amino acid oxidase derived from dead sperm (Shannon, 1965a).

The presence of an acidic heat-stable glycoprotein, named calsemin, was found in human seminal plasma by Bradley and Forrester (1982). Calsemin is a unique Ca^{2+}-dependent regulator protein. A family of four similar acidic proteins from bovine seminal plasma were reported by Chandonnet *et al.* (1990). Because of their sperm binding properties it was postulated that these proteins play a role in sperm capacitation and the acrosome reaction (Manjunath *et al.*, 1989). This theory was supported by Chandonnet *et al.* (1990). Homologous zona pellucida acidic glycoproteins in boar seminal plasma were characterised by Parry *et al.* (1992). It was suggested that they play a role in regulating the rate of sperm capacitation and survival in the female reproductive tract. Miller *et al.* (1990) found that the exposure of sperm to seminal plasma enables them, following incubation with heparin, to undergo zona pellucida-induced exocytosis of the acrosome.

Moustafa and Meszarose (1980) reported that the high protein content of bovine seminal plasma causes an increase in the permeability of sperm cell membranes and thus promotes damage to the biological membranes. Shannon (1965a) found that the addition of seminal plasma to sperm depresses viability. The sperm survived for a significantly longer period if seminal plasma was incubated previously at 37°C than when seminal plasma was stored at 5°C over the same period (Shannon, 1965b), presumably due to denaturation of this toxic factor during incubation. Incubation of boar semen for longer than 2 days at 15-18°C generally results in a marked decline in fertilising capacity (Johnson *et al.*, 1981; Johnson *et al.*, 1985).

The toxic action of seminal plasma appears to be directly on the sperm and is not a result of degradation products of components of the diluent. Vierula and Rajaniemi

(1983) showed that seminal plasma detaches the sperm surface proteins and lipids very efficiently even at a relatively low concentration. Shannon and Curson (1983) reported that the level of seminal plasma present in extended semen containing $10x10^{10}$ sperm/ml would be 1% yet the adverse effects on sperm was pronounced. It was hypothesised that the bactericidal compound(s) in bovine seminal plasma is also detrimental to sperm (Shannon, 1973). Shannon *et al.*(1974) postulated that the toxic effects of bovine seminal plasma were due to an antibacterial substance. Proteins in bovine seminal plasma may share this property (*i.e.* the antisperm-antibacterial activity). A well described example is seminalplasmin, a protein showing antibacterial as well as antisperm activity (Shivaji and Bhargava, 1987). Shannon *et al.* (1974) reported that antibacterial activity was in all fractions obtained by sequential acetone precipitation from seminal plasma. All of these fractions also reduced sperm livability.

The retentate of seminal plasma dialysed with a 12 kDa cut-off membrane significantly reduced the percent of sperm surviving cold shock, whereas the diffusate significantly increased the percent of surviving sperm. The beneficial effect of the diffusate was not apparent when sperm were cold-shocked in diluent containing both the diffusate and retentate (Shannon and Curson, 1983). The addition of retentate, combined retentate and diffusate, or whole seminal plasma to the diluents reduced the percentage of sperm surviving cold shock to much the same extent (Shannon and Curson., 1983).

1.7 Motility of sperm

Sperm motility is an important factor which should be considered when evaluating fertility. As Tampion and Gibbons (1963) stated: a man with completely immotile sperm is sterile, because immotile sperm, whether living or dead, cannot penetrate cervical mucus. However, sperm movement alone is not a sufficient criterion for fertilising capacity and lack of movement is not necessarily a sign of non-viability (Amelar *et al.*, 1980). Conflicting data have been reported on the importance of sperm motility on subsequent penetration of zona-free hamster ova. Chang *et al.* (1982) showed that human sperm with high motility penetrated more zona-free hamster ova than did sperm with lower motility. However, the majority of studies have shown that once the zona pellucida is removed, the need for sperm motility in penetrating the egg is lessened (Yanagimachi, 1984). Moreover, immotile human sperm were shown to penetrate zona-free hamster ova, suggesting that ova actually facilitate sperm penetration (Williamson *et al*, 1984; Bronson *et al.* 1985).

It has become evident that sperm motility can be beneficially or detrimentally influenced by a wide variety of physical and chemical factors. These factors include the ionic composition of the culture medium, pH, osmotic pressure, temperature, and the presence of colloids and chelating agents (Mann, 1964).

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The motility of sperm is dependent on an enzyme, dynein, that interacts with tubulin and adenosine triphosphate to generate force (Lindemann *et al.*, 1988). A change in the intra-sperm level of cAMP is important for the development of motility in the bovine epididymis since vigorous motility can be induced in caput sperm by raising cAMP levels with theophylline (a phosphodiesterase inhibitor) in the presence of seminal plasma. Following the observation that phosphodiesterase inhibitors stimulate motility of mature bovine sperm (Garbers *et al.*, 1971), and that the intrasperm cAMP content increases during epididymal maturation, attempts were made to induce motility in non-motile caput sperm by the addition of phosphodiesterase inhibitors (Hoskins *et al.*, 1975). Neither phosphodiesterase inhibitors nor seminal plasma alone could induce forward motility. Although it is not known how bovine seminal plasma stimulates forward motion in bovine caput sperm, seminal plasma has been known for some time to stimulate motility in general, and the factor responsible for the induction of forward motility in bovine caput sperm was subsequently purified and named the forward motility protein (Acott and Hoskins, 1978). Later Hoskins *et al.* (1979) reported that the requirement for theophylline is absolute; seminal plasma alone has no effect on either motility or the level of cAMP.

Since phosphodiesterase inhibitors have been shown to stimulate motility of sperm (Garbers and Kopf, 1980), the addition of caffeine (another phosphodiesterase inhibitor) to inactivated sperm has been shown to improve sperm motility. There is potential for the use of sperm stimulants in the clinical treatment of such conditions as asthenospermia and oligospermia and in the laboratory to aid semen storage by improving post-thaw motility (Taurb *et al.*, 1982). However, stimulating motility by the addition of caffeine did not improve the pregnancy rates; the most likely explanation for this seem to be the occurrence of an alteration in the ultrastructure of the sperm head as observed with a scanning electron microscopic examination after incubation of the sperm with caffeine (Harrison *et al.*, 1980). The widely documented effect of caffeine on human sperm motility is of a transitory nature with ultrastructural damage (Taurb *et al.*, 1982).

If sperm are treated with Triton X-100 to remove the cell membrane, motility can be restored in the cells with Mg-ATP (Lindemann and Gibbons, 1975; Mohri and Yanagimachi, 1980). Upon partial extraction with 1% Triton X-100, ram sperm were partially demembranated; an extensive area of plasma membrane remained intact especially in the mid-piece region (Vishwanath *et al.*, 1986). The same group reported that in the absence of ATP, of these partially demembranated sperm only 16.6% were motile, but sperm which were completely demembranated were immotile. The addition of ATP reactivated 39% of the demembranated sperm, but it reactivated 81% of the partially demembranated sperm. Partially demembranated sperm had intact mitochondria that were presumably still able to produce ATP, although the sperm movement was stimulated by exogenous ATP.

Lindholmer (1974) reported the beneficial effects of human seminal plasma in inducing forward progression in sperm from the caput epididymides of several patients with obstructive oligospermia. Rozin (1960) showed that poor sperm motility in some human patients could be improved by replacing their seminal plasma with plasma of men with normal sperm motility. This was recently confirmed by Magnus *et al.* (1991). Although several reports indicate that seminal plasma has the capacity to stimulate sperm motility (Lindholmer, 1974; Hoskins *et al.*, 1975; Inskeep *et al.*, 1985), others have demonstrated the presence of inhibitory factors (Iwamoto and Gagnon, 1988a, b). Magnus *et al.* (1991) reported that the stimulatory factor in seminal plasma was also in blood serum. This factor is suspected to be proteinaceous in nature (Hoskins *et al.*, 1979). The beneficial effects of low molecular weight components in the diffusate from bovine seminal plasma were reported by Baas *et al.* (1983) and Shannon and Curson (1983). Bavister and Yanagimachi (1977) reported that this sperm motility-stimulating component is heat stable with an estimated molecular weight of about 200 dalton.

When seminal plasma is removed by washing, sperm appear to be immotile in the same way as when semen is diluted. Motility can be restored by the addition of seminal plasma or protein fractions from seminal plasma. Other proteins can restore motility, and in the absence of seminal plasma motility may be prolonged. Breedermen and Foote (1971) and Baas *et al.* (1983) reported that replacing seminal plasma proteins with bovine serum albumin prolonged vigorous sperm motility. Working with centrifuged stallion sperm, Pickett *et al.* (1975) reported that retention of 20% seminal plasma in extended semen resulted in higher maintenance of sperm motility than retention of lower or higher percentages of seminal plasma. However,

Jasko *et al.* (1991) reported that removal of all the seminal plasma from centrifuged stallion semen improved the maintenance of sperm motility compared with that of sperm in extended semen retaining amounts of seminal plasma. But, in a further study Jasko *et al.* (1992) reported that removal of all seminal plasma from centrifuged semen extended in dried skim milk-glucose resulted in depression of sperm motility compared with that containing 5-20% seminal plasma. Conflicting data about the maintenance of motility in the presence or absence of seminal plasma may be due to differences in experimental approach in addition to the possible individual variation in the seminal plasma ability to maintain sperm motility as has been reported for men (Marden and Werthessen, 1956). Recently the influence of seminal plasma while maintaining consistent stallion sperm concentration was examined by Pruitt *et al.* (1993). They concluded that the inclusion of seminal plasma was detrimental to sperm motility, however, during short periods at 4°C and at 37°C following collection, 20% seminal plasma was not detrimental.

It was noticed by Gagnon *et al.* (1981) that low concentrations of seminal plasma interfered with the reinitiation of motility by MgATP. The human seminal plasma motility inhibitor is a minor protein and not only blocks the motility of demembranated-reactivated sperm but also that of intact human sperm (Iwamoto and Gagnon, 1988b). The seminal plasma of all mammalian species investigated so far contain a motility inhibitor (De Lamirande *et al.*, 1983, 1984). De Lamirande *et al.* (1984) commented that the seminal plasma motility inhibitor protein appears to be ubiquitous as it is present in rabbit, ram, human, boar and bull. The seminal plasma motility inhibitor also inhibits flagella movement of salmonid fish and sea urchin sperm (Okuno *et al.*, 1986). The factor inhibited the reactivated flagellar movement at a concentration of approximately 30 µg/ml (Okuno, 1987). The inhibitor is not species-specific as seminal plasma from one species inhibits the motility of demembranated sperm from a different species. This factor originates from the prostate and seminal vesicles (De Lamirande and Gagnon, 1984) in bull, rat and rabbit. In humans, this factor originates exclusively from the seminal vesicle (Luterman *et al.*, 1991).

Mammalian sperm have a very active ATP-generating metabolism. The biosynthesis and catabolism of ATP are particularly important for the motility of sperm. The interaction between sperm and seminal plasma appears to stimulate the cellular metabolism. However, this phenomenon is quite complex and is still not fully understood (Mann, 1964). It was originally thought that the immobilisation of sperm by the highly viscoelastic mucus in the epididymis serves to depress metabolism

during storage. However, Cardullo and Cone (1986) found that the metabolism of rat sperm was unaffected by immobilisation. The mature sperm cell has evolved a relatively simple metabolism, requiring energy only for the maintenance of ionic balance and motility (Inskeep and Hammerstedt, 1983). The primary exogenous energy source is simple carbohydrates, particularly fructose (Page *et al.*, 1972), so dilution of semen with a relatively simple medium containing a carbon source is admissible. It was thought that carbohydrates are involved in the initiation of sperm motility. In rat, it was revealed that the carbohydrate motility initiation requires a unique structural specificity (Tso *et al.*, 1987). Lee *et al.* (1987) reported that a sugar transport process may be involved in motility initiation.

Motility is estimated to consume over 70% of cellular ATP (Inskeep and Hammerstedt, 1983) and therefore there should be a definite cellular relationship between motility and ATP content. Components in seminal plasma which inhibit motility, such as the sperm motility inhibiting factor purified by Strzezek *et al.* (1992), significantly decreased the ATP content of sperm.

1.8 Relationship between proteins and membrane

It appears that the proteins in seminal plasma exert their biological activity by binding to a specific region on the sperm membranes. This binding must involve various factors to enable a strong relationship between the protein and the sperm membrane. The relationship between some of the proteins in seminal plasma, such as the anti-fertility protein and the sperm membrane are strong enough to prevent the removal of this factor from the sperm by washing (Reddy *et al.*, 1982; Audhya *et al.*, 1987). This strong relationship was attributed to the carbohydrate nature of the anti-fertility protein (Reddy *et al.*, 1982; Audhya *et al.*, 1987).

Proteins in seminal plasma bind to sperm through carbohydrate bridging. For example the decapacitation factor which consists of a protein-carbohydrate complex is reported to be bound to the sperm head by a sialic acid unit (Dukelow *et al.*, 1966). The epididymis-specific proteins in rat, which were reported by Brooks (1981a, 1981b, 1982) and confirmed by Shabanowitz and Killian (1987), are bound to the plasma membrane of rat cauda sperm in the acrosomal and postacrosomal regions (Cameo *et al.*, 1986).

The major proteins of bovine seminal plasma, BSP-A1, BSP-A2, BSP-A3, and BSP-30 kDa (Manjunath and Sairam, 1987; Manjunath *et al.*, 1987; Seidah *et al.*, 1987; Bányai *et al.*, 1990) bind to sperm upon ejaculation (Manjunath *et al.*, 1988). Studies

have revealed that they bind to apolipoprotein A-I (Manjunath *et al.*, 1989), the major protein of high-density lipoproteins. They also bind to phospholipids containing choline which represent more than 73% of the total lipids present in the plasma membrane of bovine sperm (Pursel and Graham, 1967; Clegg and Foote, 1973). These phospholipids constitute the binding site of the major proteins on the surface of the sperm membrane (Desnoyers and Manjunath, 1992). Recently, Leblond *et al.* (1993) showed that seminal plasma from six species have seminal plasma proteins which possess an affinity for choline-containing phospholipids.

There is a specific binding site on the surface membrane of sperm for several proteins in seminal plasma. For example, there is the "major protein" reported by Aumüller *et al.* (1988) which specifically binds to the mid-piece of the sperm tail and to the neck region, and the calcium-binding protein caltrin/seminalplasmin (Shivaji, 1986) which presumably binds to calcium porters located in the region of the plasma membrane over the acrosome and the principle tail (San Agustin and Lardy, 1990). Immunochemical studies by Aumüller *et al.* (1988) revealed the specific binding of the "major protein" to the fossa and the mid-piece region of the tail of bull sperm. The binding of gold-labelled "major protein" to subplasmalemmal sites at the mid-piece region of sperm was demonstrated by electron microscopy and two membrane proteins which selectively bind the major protein were isolated from bull epididymal sperm (Aumüller *et al.*, 1988).

1.9 Anionic and cationic proteins and peptides and their effects on sperm

Analysis of seminal plasma revealed the presence of a large number of proteins. However, it is not clear if they all have a significant biological role. The effects of the various protein or peptide components in seminal plasma may be influenced by their ionic charges. The net charge on the protein or the peptide influences its interaction with other components in the seminal plasma and the sperm membrane. These interactions may be vital for a certain physiological functions.

The seminal secretions are a complex of acidic and cationic proteins. The composition of bull semen at pH 7 was 55% anionic protein, 30% neutral protein, and 15% cathodic migrating protein (Jeyendran *et al.*, 1983). When rabbit seminal plasma was subjected to SDS polyacrylamide electrophoresis, 14 bands staining for protein were consistently observed (Reyes *et al.*, 1975). The molecular weight of these components after dissociation with SDS ranged from less than 12 kDa to 200 kDa (Reyes *et al.*, 1975; Yantorno *et al.*, 1972). Lavon and Boursnell (1971) suggested that up to 22 components of seminal plasma may be separated by disc gel

electrophoresis and between 35 and 40 components by isoelectric focussing. These components have been focussed in the acidic and the basic region.

It was demonstrated by Brooks (1983) and Kohane *et al.* (1983) that there are at least four anionic proteins specific to the epididymis in rats. Shabanowitz and Killian (1987) studied the cauda epididymal fluid of rat. They used two-dimensional electrophoresis under reducing conditions which indicated the presence of 32 proteins. The molecular weights of these proteins ranged from 15 to 67 kDa and most of them were in the acidic region with isoelectric point between 4.9 and 6.2. The presence of another 20 proteins with molecular weights ranging from 11 to 66 kDa and isoelectric points between 5.4 and 7 were indicated, using two-dimensional electrophoresis, from the caput and the corpus epididymis (Shabanowitz and Killian, 1987). Rabbit cauda epididymal fluid contains four main acidic glycoproteins (pI between 3.45 and 4.9) as determined by isoelectric focussing (Moore, 1980).

Boar epididymal plasma contains 10 proteins, as determined by electrophoresis (Lavon and Boursnell, 1971). Two dimensional electrophoresis of the epididymal fluid of boar revealed the presence of 175 major polypeptides with molecular weights and isoelectric points ranging between 12-300 kDa and pH 3.5-8.5 respectively (Russell *et al.*, 1984). Bovine epididymal fluid resolved into 17 bands upon electrophoresis which contains at least eight bands specific to the epididymal region (Amann *et al.*, 1973). The presence of three proteins, with molecular weights of 94, 78 and 24 kDa, which are unique to the epididymis in ram, were reported by Voglmayr *et al.* (1980, 1982).

When rat seminal vesicle secretion was subjected to SDS-PAGE under reducing conditions, 7 distinct bands were seen with molecular weights ranging between 15 and 80 kDa were mostly acidic in nature (Fawell *et al.*, 1987). Guinea pig seminal vesicle secretion was resolved under similar conditions into eight proteins; only one of these proteins was cationic (pI 9.3) and appeared to have a molecular weight of approximately 18 kDa (Williams-Ashman *et al.*, 1972).

The major protein in bovine seminal vesicle secretion appears to be acidic in nature with a molecular weight of 16 kDa (Kemme *et al.*, 1986). Aumüller *et al.* (1988) reported that the bovine seminal vesicles synthesise a neutral protein with molecular weight of 13.5 kDa. This protein was found to make up 40% of the seminal vesicle secretion. Bovine seminal plasma forward motility protein was reported to be separated into six major activity peaks with isoelectric points between 4 and 7.

The acidic proteins from bovine seminal plasma reported by Manjunath and Sairam (1987) were found to be identical to those proteins isolated from bovine seminal plasma by Esch *et al.* (1983), which were designated BSP I, BSPII and BSPIII. These acidic proteins were reported to make up 30% of the total proteins in bovine seminal plasma. Later Chandonnet *et al.* (1990) reported that a group of four similar proteins represent the major acidic proteins found in bovine seminal plasma and they were designated as BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa. The stimulatory and inhibitory action of the acidic proteins on the release of pituitary gonadotropins was reported (Manjunath, 1984). The acidic proteins reported by Manjunath (1984) and Chandonnet *et al.* (1990) appeared to have a cluster of cationic amino acids which act in a similar manner to that reported by Cardin and Weintraub (1989). These clusters form centres of high positive charge density which electrostatically interact with the acidic groups of the glycosaminoglycans, such as heparin, which is present in the female reproductive tract and thus is involved in capacitation of sperm. In a recent report, Kononov *et al.* (1991) observed that some of the anionic fractions from bovine seminal plasma decreased the duration of motility of washed bull sperm.

It was reported that amongst the acidic proteins in bull seminal plasma, there are three proteinase isoinhibitors with pI values of 3.4. 3.7, and 3.9 (Cechová *et al.*, 1979a, b). Strzezek *et al.* (1992) reported the purification of an anionic peptide from boar seminal plasma, with a molecular weight of 5.7 kDa. Acidic glycoproteins in boar seminal plasma were characterised by Parry *et al.* (1992). These proteins were described as having relatively low molecular weights, ranging from 16 to 23 kDa. Sanz *et al.* (1993) isolated heparin-binding proteins from boar seminal plasma. Among the proteins identified, a boar counterpart of bovine seminal plasma capacitation factors BSP-A1/2 and PDC-109 (acidic proteins) which were isolated by Chandonnet *et al.* (1990) and Esch *et al.* (1983). Remarkably, all the heparin-binding proteins reported by Sanz *et al.* (1993) had a molecular weight between 12-18 kDa. In human seminal plasma, the presence of an acidic heat-stable glycoprotein with a molecular weight of 16.5 -20 kDa and a pI of 4 was reported by Forrester and Bradley (1980). Acidic proteins called calmodulin and calmodulin-like have been shown to be present in mammalian sperm and seminal plasma (Weinman *et al.*, 1986; Sidhu and Guraya, 1993). These proteins are believed to be involved in regulation of calcium levels in the sperm and are implicated in the sperm acrosome reaction (Sidhu and Guraya, 1993).

The majority of the protein components in the seminal plasma of the boar are secreted by the seminal vesicle which has a high concentration of cationic proteins (Boursnell *et al.*, 1962). Boar seminal vesicle secretion was shown by chromatography on Sephadex G-200 (under non-dissociating conditions) to contain two major basic protein components (Boursnell *et al.*, 1966). Three principle components are involved, two of which are positively charged and have isoelectric points of 8.8 and 9.4 (Boursnell and Briggs, 1969). Boursnell (1967) demonstrated the ability of boar, bull and ram sperm to adsorb basic proteins from porcine seminal plasma.

Bovine seminal plasma has been reported to have cationic proteins (Shannon *et al.*, 1987) and acidic proteins (Manjunath and Sairam, 1987). The cationic proteins were reported to be responsible for the sperm damaging activity (Shannon *et al.*, 1974; Baas, 1985). A report from Hibbitt and Benians (1971) indicates that boar seminal plasma contains cationic proteins which can increase the permeability of biological membranes. Recently, Iwamoto *et al.* (1992) reported that the motility inhibitor they purified from boar seminal plasma is a cationic protein with a pI between 8.7 and 9.1. Bull seminal plasma contains a proteinase inhibitor with a pI of 10.5 and a molecular weight of 6.2 kDa (Cechová *et al.*, 1979a). The heparin binding proteins were isolated by heparin affinity chromatography from the seminal plasma of vasectomized bulls. Gel electrophoresis indicated that the heparin-binding peaks contained 14-18 kDa proteins with various isoelectric points, a cationic 24 kDa protein and a 31 kDa protein (Miller *et al.*, 1990).

A cationic protein, believed to be a dimer of two polypeptides with molecular weights of 20 kDa, was isolated from bull seminal vesicle fluid (Dostal and Matousek, 1972). In 1986, Scheit reported that all the cationic proteins present in the secretions of the seminal vesicles of bull could be resolved into four components on a reverse phase column.

Seminalplasmin is cationic protein with antimicrobial activity isolated from bovine semen (Reddy and Bhargava, 1979, Shivaji, 1984). It has been estimated to have a molecular weight between 8 and 19.8 kDa depending on the method used. It is heat-stable and has an isoelectric point of 9.8. Theil and Scheit (1983) have subsequently reported the isolation of pure seminalplasmin with a molecular weight of about 6.3 kDa.

Preliminary studies indicate that seminalplasmin inhibits the growth of both bacteria and yeast by inhibiting the synthesis of RNA (Scheit and Bhargava, 1985; Scheit *et*

al., 1985). As seminalplasmin has antimicrobial and reverse transcriptase-inhibitory activity, it could protect the male and/or the female reproductive tracts against micro-organisms and retroviruses (Shivaji, 1988).

Shivaji (1986) studied the effects of seminalplasmin on the plasma and acrosomal membranes of bovine sperm. It was found that seminalplasmin has detrimental effects which leads to an increase in the fluidity of both membranes. This protein inhibits the motility and acrosome reaction due to blockage of the entry of Ca^{2+} essential for this reaction and fertilising ability of bull sperm (Shivaji, 1988; Shivaji and Bhargava, 1987). This protein is secreted only by the accessory sex glands, indicating that it interacts with sperm for the first time only at the time of ejaculation (Shivaji *et al.*, 1984).

Another protein called antiseminalplasmin has been purified from bovine seminal plasma. It is capable of reversing the inhibitory effects of seminalplasmin, not only on the growth and synthesis of RNA and growth of *E. coli*, but also on the motility and the fertilising ability of sperm (Rao and Bhargava, 1985). Antiseminalplasmin is a weakly acidic glycoprotein with a minimum molecular weight of 39 kDa.

The seminalplasmin-antiseminalplasmin system may have evolved as a means of regulating fertility. If, like with seminalplasmin, sperm are exposed to antiseminalplasmin for the first time only at the time of ejaculation, the chance of a particular sperm encountering seminalplasmin or antiseminalplasmin first would depend on the relative concentrations of the two proteins in semen, which may be under fine humoral/endocrine or other (such as nutritional or emotional) control. If a sperm gets coated with seminalplasmin first, it may no longer be in a position to eventually fertilise an egg, as it may not be able to undergo the acrosome reaction. If the cell gets coated with antiseminalplasmin at irrelevant sites, this would not affect its fertilising capacity. This would account for the two patterns of distribution of seminalplasmin observed on the surface of ejaculated sperm (Bhargava, 1986).

Caltrin is a bovine seminal plasma protein of seminal vesicle origin (Shivaji *et al.*, 1984), with a molecular weight of 5411 dalton (Rufo *et al.*, 1982; Shivaji *et al.*, 1984). It was shown by Lewis *et al.* (1985), that caltrin, upon comparison of amino acid residues, is identical to seminalplasmin. The initial role of caltrin is the prevention of premature calcium uptake by freshly ejaculated sperm (Shivaji *et al.*, 1984).

1.10 Protein transformation and protein aggregates in seminal plasma

It is well known that many seminal plasma proteins are transformed rapidly into small proteins immediately after ejaculation (Montagnon *et al.*, 1985). Although freshly isolated caltrin initially behaves as an inhibitor of calcium uptake in bovine sperm (Rufo *et al.*, 1982), it subsequently undergoes a remarkable transformation with time to become an enhancer of calcium uptake.

Protein stability in an aqueous medium is dependent in part on its bound water content (Taborsky, 1979). Removal of this water can lead to alterations in the protein structure and function (Kuntz, 1971). Jeyendran *et al.* (1983) reported that upon freeze-drying of bovine semen (residual moisture was less than 6%), the percentage of cationic proteins and neutral proteins decreased with a concurrent increase in anionic migrating proteins on agar gel electrophoresis.

Some of the proteins in seminal plasma appear on analysis to be closely related both in molecular weight and charge. There have been various reports of a vast number of bands appearing upon isoelectric focussing, indicating that there are at least some components which are similar in molecular weight with slight differences in charges. On the other hand, while gel permeation chromatography may reveal a small number of peaks, further analysis by electrophoresis reveals a large number of components. On some occasions, components have been found to consist of several subunits. This property appears not to be specific to any one species of animal. The common protein pattern of seminal plasma among the various species does not exclude species specificity. Bennett (1965), in a quantitative comparison of the proteins of the seminal plasma of bull, ram, rabbit and boar, reported that typical patterns were obtained on electrophoresis of seminal plasma. Glycoproteins and sialic acid were found to be present in all the seminal plasma samples. Lipoproteins were present in small but variable amounts in the seminal plasma of rabbit, bull and boar but were apparently absent in ram seminal plasma. Rabbit and boar seminal plasma each contained one lipoprotein, while bull seminal plasma contained four or five. Bennett (1965) also reported little variation in the protein pattern between individual animals of one species, in contrast to the striking species specificity.

From the various reports in the literature it appears that many of the described proteins have similar molecular weights and functions yet have been named differently. In other reports, the molecular weight of the protein described is dependent on the determination method. While electrophoresis gives an estimated molecular weight for a substance, gel permeation chromatography gives a different

estimation of the molecular weight. In other reports gel permeation chromatography under acidic and neutral conditions gave a different pattern for the same substance. Some of these proteins re-aggregate to a higher molecular weight when chromatographed at neutral pH.

Upon gel permeation chromatography, proteins appeared to have a molecular weight which differed when estimated by electrophoresis under reducing conditions in the presence of SDS. Eng and Oliphant (1978) and Reynolds and Oliphant (1984) reported the isolation of an acrosome stabilising factor with a molecular weight of 355-370 kDa from rabbit seminal plasma. This protein was found to be in a native monomeric and dimeric relationship, each monomeric unit being composed of subunits. Later Thomas *et al.* (1986) reported the characterisation of the factor by SDS electrophoresis. This demonstrated that there were two main subunits with estimated molecular weights of 38 and 92 kDa. Lavon and Boursnell (1971) reported that boar seminal plasma, vesicular secretion and epididymal plasma proteins gave fifteen to twenty bands in gel disc electrophoresis and that larger numbers of bands were revealed (especially in the acidic region) with isoelectric focussing than were ever found by electrophoresis.

The presence of a high molecular weight anti-fertility factor in human seminal plasma was established by Reddy *et al.* (1979). The molecular weight of this factor (by SDS electrophoresis) was found to range from 40 to 200 kDa. This factor is thought by Reddy *et al.* (1979) to consist of several subunits of low molecular weight tightly adhered to high molecular weight material, which can be reduced to the individual components by treatment with mercaptoethanol and SDS. In 1982, Reddy *et al.* reported the isolation of this factor from human seminal plasma in a high degree of purity. Audhya *et al.* (1987) reported the purification and partial chemical characterisation of a glycoprotein (AF-1) with anti-fertility activity from human seminal plasma. This factor appeared to be similar to that reported by Reddy *et al.* (1979). It has a molecular weight of about 200 kDa and it consists of two subunits with molecular weight of about 125 and 72 kDa.

Upon electrophoresis in the presence of SDS the motility inhibitor from boar seminal plasma reported by Iwamoto *et al.* (1992) appeared to be composed of three subunits with molecular weights from 14-18 kDa, however, when chromatographed under neutral conditions the motility inhibitor behaved as a protein eluting as a single peak with a molecular weight of about 50 kDa.

Recently Kameda *et al.* (1992) showed that gel permeation chromatography of human seminal plasma at neutral pH gave a peak of antigenic activity with an estimated molecular weight greater than 669 kDa. But when chromatography was conducted at neutral pH in the presence of SDS the whole pattern changed with a shift to lower molecular weights occurring and the antigenic activity appearing in a peak corresponding to a molecular weights of approximately 15 kDa while the peak of high molecular weight shrank. It was suggested that the protein of interest may form an aggregate with itself.

Ionic interactions between the proteins in seminal plasma appears to hold them together in aggregated forms. When the ionic interaction between proteins is affected, the high molecular weight components appear to transform into low molecular weight ones. Davies *et al.* (1975) reported that chromatography of boar seminal plasma on Sephadex 200 at pH 4 resulted in most of the proteins being eluted in the relatively low molecular weight area indicating that at acidic pH many proteins in seminal plasma dissociated into smaller subunits. Manjunath and Sairam (1987) found that a peak which corresponded to a molecular weight of about 15-20 kDa, obtained by chromatography of anionic material from bovine seminal plasma on Sephadex G75 under acidic conditions, upon rechromatography on Sephadex G200 at pH 8.5 yielded three peaks with estimated molecular weights ranging from 20 to 120 kDa. Dacheux *et al.* (1983) reported that when an antiagglutination protein isolated from boar epididymal plasma by fractionation of epididymal fluid on Sephadex G100 was chromatographed on DEAE-Sephacel, it was resolved into seven distinct peaks.

Bovine seminal plasma forward motility protein was reported by Acott and Hoskins (1978) to be present in all peaks upon fractionation of bovine seminal plasma Sephacryl 300 column. Acott and Hoskins (1978) found that the activity spread over a wide range of molecular weight ranging between 10 kDa and 1.6×10^6 Da. Changes in the ionic strength of the eluting buffer on the gel permeation chromatography column changed the relative amounts of activity found in each of the four peaks. Elution at low ionic strength produce more of the high molecular weight form, while elution at higher ionic strength shifted the activity toward the lower molecular weight peaks. Acott and Hoskins (1978) provided further evidence of an aggregate equilibrium among the proteins upon the rechromatography of each of the individual peaks which resulted in the formation of four new peaks of activity. More significantly, preincubation in dissociating buffers (*i.e.* in the presence of SDS or urea) at 37°C and subsequent gel permeation chromatography produced a single major peak of forward motility protein. When the dissociating conditions were

removed, the rechromatography of this peak produced the same four peaks of activity as seen originally. It can be concluded that the forward motility protein exists in aggregation-disaggregation equilibrium of high and low molecular weights. SDS electrophoresis revealed several proteins with molecular weights between 11 and 17 kDa.

De Lamirande *et al.* (1984) reported that the motility inhibitor protein appeared in the diffusate when seminal plasma from rabbit, ram, human, boar, and bull was dialysed at pH 4, suggesting that ionic interactions held a motility inhibitor factor in the form of an aggregate which dissociated to release this motility inhibitor when breakdown of the holding forces took place since re-dialysis of the retentate produced more of the motility inhibitor.

The existence of proteins in the form of aggregates have been reported by Shannon *et al.* (1987). They demonstrated that the antibacterial activity had passed through an ultrafilter with a nominal cut-off of 2 kDa. The diffusate, the components of which would have an apparent molecular weight of 2 kDa or less, when chromatographed on a Sephadex G50 column at pH 3 produced a peak in the higher molecular weight range, however, the most prominent peak was in the area of low molecular weight. But at pH 7, the elution profile was different in that all the components were eluted in the relatively high molecular weight range (greater than 10 kDa) and one peak at the void volume indicating a molecular weight of 20 kDa or greater. In a similar study it was found that the fraction which eluted in the region of 15 to 20 kDa under acidic conditions aggregated and eluted in several peaks up to 120 kDa when chromatographed on G200 under neutral conditions (Manjunath and Sairam, 1987).

A large proportion of the protein in bovine seminal plasma exists aggregated in macromolecules of about 500 and 250 kDa (Hameed *et al.*, 1991). The proposal that low molecular weight peptides are associated with macromolecular protein fractions of seminal plasma (Parry *et al.*, 1992) may be due to the dynamic system of aggregation/disaggregation observed by Shannon *et al.* (1987).

1.11 The effect of low molecular weight proteins and peptides in seminal plasma on sperm

The damaging effects of high-molecular weight non-dialysable materials found in bovine seminal plasma on sperm motility have been studied by a number of researchers (Baas *et al.*, 1983; Schmehl *et al.* ,1986; Garcia and Graham, 1987a and b). Seminal plasma fractions with anti-sperm activity have been described as having a

wide range of molecular weights and charges. Baas *et al.* (1983) indicated that the anti-sperm activity is associated with proteins of molecular weight higher than 10 kDa. Collingwood (1990) indicated that the bulk, if not all, of the sperm damaging components of seminal plasma are present in the fraction of high molecular weights . It was reasoned that the active components themselves are either of high molecular weight or consist of an aggregation of low molecular weight components. However, components with a molecular weight of 10-12 kDa were implied in the anti-sperm activity such as the motility inhibitor of boar and bull sperm reported by De Lamirande *et al.* (1984). Iwamoto and Gagnon (1988a) reported that human seminal plasma contains motility inhibitor with a molecular weight of 15-18 kDa. In a recent report, Iwamoto *et al.* (1992) purified a motility inhibitor from boar seminal plasma with a molecular weight of 14-18 kDa. Recent studies showed that seminal plasma contains low molecular weight substances which interfere with the fertility and motility of sperm and have many biological activities. A group of relatively low molecular weight (16-23 kDa) glycoproteins with haemagglutination activity and a high affinity for homologous zona pellucida glycoproteins were isolated from boar seminal plasma (Parry *et al.*, 1992). An acidic 5.7 kDa motility inhibitor peptide was isolated from boar seminal plasma (Strzezek *et al.*, 1992). The peptide also showed antibacterial activity against Gram-positive bacteria and lytic activity against *Micrococcus lysodeikticus* suggesting interaction with the sperm membrane (Strzezek *et al.*, 1992). Tareda and Watanabe (1980) observed that the low molecular weight fraction from cock seminal plasma exerts an adverse effect on frozen-thawed sperm, but the macromolecular fraction (>10 kDa) does not produce harmful effects on either the motility or the morphology of sperm.

It has been reported that the low-molecular-weight fraction (<12 kDa) found in dialysates from seminal plasma has adverse effects on sperm survival in fresh and frozen bovine semen (Vasquez and Graham, 1980; Garcia and Graham, 1987a and b). An improvement in post-thaw motility of bovine sperm was obtained when the low molecular weight fraction from seminal plasma was removed by dialysis (Schmehl *et al.*, 1986; Garcia and Graham, 1987a, b).

Dialysis experiments have been used (Collingwood, 1990; Baas, 1985) to separate low molecular weight and high molecular weight components in seminal plasma. Dialysis is also used in processing of semen to introduce a low molecular weight cryoprotectant. Many reports indicate that dialysis benefits sperm survival apparently by removal of detrimental low molecular weight components (Shannon and Curson, 1983). De Lamirande *et al.* (1984) reported that the motility inhibitor protein present

in the seminal plasma of rabbit, ram, human, boar and bull appeared in the diffusate when seminal plasma from all these species was dialysed at pH 4. Further dialysis of the retentate gave more motility inhibitor in the diffusate, however, the period of dialysis was not stated in this report. Low molecular weight components in bovine seminal plasma with detrimental effects on sperm were also reported by Garcia and Graham (1987a, b). They reported that dialysis for a period of 2 hours removed these components. This removal apparently improved the survival of sperm. However, Shannon and Curson (1983) reported that whereas there was a beneficial effect of dialysis for a short period, dialysis for a longer period caused more detrimental effect to sperm survival.

Low molecular weight antibacterial peptides (2.2 kDa, Eschenbruch, 1980; <2 kDa, Shannon *et al.*, 1987; 1.2 kDa , Hameed, 1987) were isolated from bovine seminal plasma. The anti-sperm activity of these antibacterial substances was not studied although the report of Shannon *et al.* (1974) suggested that the antibacterial fractions in bovine seminal plasma exerted anti-sperm activity.

1.12 Aim and approach of this investigation in this thesis

From the data reviewed, it is clear that seminal plasma has both beneficial and detrimental effects on the viability of sperm. There is still clearly some debate about the size and the nature of the detrimental and the beneficial component(s). The antibacterial aggregates of various molecular weights, their components and their contribution to the anti-sperm activity are still not clear. The aggregates are reported to be held by two main forces: ionic and hydrophobic interactions (Hameed, 1987). It is not known how much each component contributes to the aggregates to form the most active fraction(s), the nature of interactions between the components (*i.e* active and inactive forms) is not clear.

The main aim of this study was to further investigate the detrimental effects, to identify and isolate the anti-sperm component(s) in bovine seminal plasma, to examine the possibility that antisperm activity exists in aggregates similar to those reported for the antibacterial activity and to find if the active component(s) can be separated from the inactive one(s). Other aims of this study were to determine whether the detrimental component(s) are of low or high molecular weights, anionic or cationic and to develop a reliable and reproducible method for isolating these components. It was hoped that purification and characterisation of these components of bovine seminal plasma could further add a piece of information important to help the development of methods for reduction of the detrimental effects of seminal plasma on

sperm and to contribute to the viability of sperm in storage for the purposes of artificial breeding.

CHAPTER 2
MATERIALS AND METHODS

2.1 Bovine semen and seminal plasma

Bovine semen was collected by the use of an artificial vagina from the top quality Friesian and/or Jersey bulls at the Livestock Improvement Corporation., Newstead, New Zealand. The artificial vagina, lubricated with vaseline, was drained into a glass centrifuge tube maintained at 38°C. The centrifuge tube, kept in insulated container, was transported to the research lab and processed within 15 min after collection. For each study semen was pooled from at least three bulls. Ejaculations with less than 75% of the sperm motile were rejected.

Bovine seminal plasma was obtained from two sources: from fresh semen (processed within 10 min) by centrifugation at 1200 *g* (to remove sperm and large particles) followed by centrifugation at 20 000 *g* for 10 min (to remove any particles before injecting the sample on FPLC columns), and from vasectomized bulls. In both cases an artificial vagina was used to obtain semen or seminal plasma pooled from a number of different bulls. Seminal plasma was either stored at -20°C until required, or at 4°C if to be processed within 1 h. All seminal plasma samples were filtered through a 0.2 µm filter prior to use.

2.2 Determination of sperm concentration

The concentration of the sperm in semen was determined by diluting a sample of the semen 10-fold into a solution containing 2% formaldehyde in saline. The absorbance of the solution at 580 nm was then measured in a spectrophotometer against a blank of 2% formaldehyde in saline. The spectrophotometer was previously calibrated against counts carried out in a haemocytometer.

2.3 Dialysis of bovine semen and seminal plasma

Dialysis was carried out at 4°C in dialysis tubing with a molecular weight cut-off of 10-12 kDa (Visking Co. Chicago, Illinois, USA), or 3.5 kDa or 1 kDa (SPECTRA/POR, Spectrum Medical Industries, Los Angeles, California, USA).

2.4 Semen diluents

14G: 2% (w/v) tri-sodium citrate, 0.3% (w/v) glucose, 2.5% (w/v) glycerol, 1% (w/v) glycine, 2.5 mmol/l $CaCl_2$, 2.2 mmol/l $MgSO_4$, 3.5 mmol/l K_2HPO_4, 0.025 g/l sodium sulphacetamide, 1.25 x 10^6 units/l Penicillin, 1.25x 10^6 units Streptomycin, at pH 6.9.

DS14G: as 14G, except double strength.

2.5 Washing of sperm

Washing the sperm to remove seminal plasma was carried out by following the method of Baas *et al.* (1983) which was based on the technique of Harrison (1976). Freshly ejaculated semen was diluted 1:1 in 14G diluent, 2 ml of the diluted semen was then carefully layered onto the top of 8 ml 7% Ficoll solution in 14G diluent in a 10 ml plastic tube. It was then centrifuged at 700 *g* for 12 minutes. The supernatant, which contained the seminal plasma, was aspirated from the sperm pellet which was then resuspended in 14G diluent to a final concentration of approximately 11-13x10^6 sperm/ml.

Bovine serum albumin was included at 0.1% concentration to aid the movement of the washed sperm and it was included in all experiments that involved washed sperm since washed sperm tend to stick to the glass microscope slides.

2.6 Motility assessment

The assessment of sperm motility was carried out visually using a microscope. A drop of the sperm suspension in 14G solution pre-heated to 37°C was placed on a glass slide resting on a heated microscope stage at 37°C. It was then covered with a pre-heated coverslip. The estimation of motility, as the percentage of motile sperm, was from five fields. The mean of five estimates was recorded.

2.7 Antibacterial assay

Colonies of *Escherichia coli* from blood agar plates were used to inoculate phials of trypticase soy broth which were then incubated at 37°C for 18 hours . The agarose medium was made up in batches of 1.6 g nutrient broth and 2.0 g agarose with 200 ml R/O purified water, brought to boil, autoclaved and cooled to 45°C in a waterbath. Between 200 and 500 µl of the 18 hour culture was added aseptically, then the agar was agitated and poured into petri dishes. Approximately 10 plates per 200 ml of agar were obtained.

The plates were stored for up to two weeks at 4°C. Holes 8 mm in diameter were cut using a cooled flamed cork borer in the plates. These were filled with 100 µl of test solution. The plates were incubated for about 18 hours at 37°C. A clear zone was produced by the lack of growth over the area affected by the tested samples which was proportional to the antibacterial activity. Activity was taken as the square of the radius of the clear zone (in mm^2), *i.e.* the square of the distance measured from the well centre to the edge of the cleared zone.

2.8 Testing for antisperm activity

To 900µl of washed sperm suspended in 14 G diluent, 100µl of the fraction of seminal plasma under test (in 14G diluent) was added to give 10% concentration of the fraction and a sperm concentration of 10-12 x 10^6 sperm/ml. The mixture was placed in 10 ml polycarbonate test tubes (in glass tubes sperm tend to stick to the surface).The motility of sperm was assessed immediately after addition and after 18 hours incubation at ambient temperature (18 to 21°C).

2.9 Gel permeation chromatography

Gel permeation chromatography was carried out with Sephadex G10, G15 and G25SF (Pharmacia, Uppsala, Sweden) using the following Pharmacia chromatography columns: K26 column with an internal diameter of 2.6 cm and length of 35 cm, XK16 columns with an internal diameter of 1.6 cm and length of 70 cm, and XK16 of internal diameter of 1.6 cm and length of 20 cm. These columns were prepared and used according to the manufacturer's instructions. Void volumes (V_o) and bed volumes (V_t) are indicated in the figures showing the elution profile in each individual experiment. The flow rate and the composition of the elution buffers are described along with each individual experiment.

2.10 Ion exchange immobilisation

Ion exchange immobilisation was performed using a Pharmacia chromatographic column K26 with an inner diameter of 2.6 cm and a length of 35 cm, and XK50 with an inner diameter of 5 cm and length of 20 cm. SP Sephadex C-25 in the sodium form, equilibrated with the starting buffer, was used for immobilisation of basic proteins and peptides. The eluents that were used with the ion exchanger were: 30% acetonitrile made to pH 3 with HCl and 0.1 mol/l of NaOH (pH 13). These are specified with each experiment.

2.11 FPLC (fast protein liquid chromatography)

A Pharmacia FPLC system was used with an LKB UVM 2141 variable wavelength monitor with simultaneous detection at two wavelengths. The sensitivity (absorbancy range) of the monitor was adjusted according to the nature of the loaded sample. The other parameters were adjusted according to the experiment. These are stated with the individual experiment.

All FPLC columns were supplied by Pharmacia (Uppsala, Sweden)

2.11.1 Gel permeation chromatography:

A) Preparative Superose 12 in a column of 1.6 cm inner diameter, 51 cm length, and 100 ml volume (the recommended molecular weight range being 1-300 kDa).

B) Analytical Superose 12 HR 10 in a column of 1 cm inner diameter, 30 cm length, and 20 ml volume (the recommended molecular weight range being 1-300 kDa).

The following proteins were used as marker proteins (Sigma Chemical Company): IgG (165 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine GH (22 kDa) and insulin (6 kDa).

2.11.2 Hydrophobic interaction chromatography:

Phenyl Superose HR 5/10 with a phenyl functional group, in a column of 0.5 cm inner diameter and 10 cm length.

2.11.3 Reversed phase chromatography:

ProRPC HR 5/10 in a column of 0.5 cm inner diameter and 10 cm length for separation of proteins <500 kDa and PepRPC 5/5 in a column of 0.5 cm inner diameter and 5 cm length for separation of peptides of molecular weight <6 kDa.

2.11.4 Ion exchange chromatography:

Mono Q HR 5/5 (anionic exchanger) and Mono S HR 5/5 (cationic exchanger), each in a column of 0.5 cm inner diameter and 5 cm length.

When monitoring at high sensitivity, any contaminants could cause spurious peaks, especially plasticiser material. Consequently, the syringe and test tubes used to handle samples were made of glass.

Buffers used in each individual experiment are described along with the other data in each experiment.

2.12 Concentration of fractions relative to seminal plasma

Assuming the recovery of the protein in each purification step was 100% (which is practically not the case), the relative concentration of the plasma fractions obtained from fractionation of seminal plasma was calculated as a percentage of the original concentration in seminal plasma. Comparison of the final volume of a sample with the volume of the whole seminal plasma from which it was fractionated was used to

determine the degree of dilution or concentration of the fraction that was necessary to adjust it to its original concentration.

2.13 Determination of protein concentration

The concentration of protein in plasma fractions was determined by using the Biuret method (Gornall *et al.*, 1949). Bovine serum albumin was used as a standard.

2.14 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed using a Pharmacia LKB BROMMA 2050 MIDGET Electrophoresis Unit with electrophoresis power supply EPS 500/400. Vertical SDS-discontinuous PAGE was performed according to the Laemmli (1970) method. For the low molecular weight fractions, the gel system of Giulian *et al.* (1985) was used- with a 20% acrylamide separating gel and a 10% stacking gel, which both contained 10% glycerol. Molecular weight markers from Bio-Rad (Bio-Rad Laboratories Ltd California, USA), BDH (BDH Chemicals Poole, UK), and Boehringer Mannheim GmbH (Mannheim W. Germany) were used. The following proteins were used as marker proteins: phosphorylase B (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.5), cytochrome c (12.5 kDa), aprotinin (6.5 kDa), insulin B chain (3.4 kDa), and bacitracin (1.45 kDa). Gels were fixed in 50% ethanol-10% glacial acetic acid for 30 minutes, then transferred to 10% gluteraldehyde solution overnight for cross-linking before they were washed with two litres of water according to the method of Giulian *et al.* (1983). The gels were then stained with either 0.02% of Coomassie R-250 (Pharmacia, Sweden) or silver staining using a Bio-Rad kit (Bio-Rad Laboratories Ltd California, USA) following the procedure prescribed by the manufacturer.

A Pharmacia PhastSystem was used also for gel electrophoresis. A PhastGel was used with PhastGel SDS buffer strips as detailed in the Pharmacia PhastSystem Separation Technique File No.100. The buffer system in the gel consisted of 0.112 mol/l acetate and 0.112 mol/l Tris, pH 6.4. The buffer strips consisted of 0.2 mol/l tricine, 0.2 mol/l Tris and 0.55% SDS, pH 8.1, in a matrix of 3% agarose.

When the protein concentration of the sample was low, a 5x concentrated sample buffer was used to allow a less diluted sample to be loaded onto the gel.

The samples were prepared for electrophoresis by diluting them 4-fold into 10 mmol/l Tris buffer (pH 8) containing 1 mmol/l of EDTA, 2.5% SDS and 5%

mercaptoethanol. They were then heated at 100°C for 6 minutes, followed by cooling down to room temperature before 0.01% of bromophenol blue was added. The same procedure was followed for the molecular weight standards.

Pre-cast gels from Pharmacia were used with an acrylamide concentration of 15% or gradient gels from 8 to 25% acrylamide. A PhastGel High Density gel was used for separation of peptides with molecular weight below 20 kDa. The gel contained 30% ethylene glycol to increase resolution of proteins with molecular weights below 10 kDa.

Aliquots containing 1 µl of each sample were applied to the PhastGel and were separated at 15°C with a voltage of 250 V and a current of 10 mA.

2.15 Isoelectric focussing

A Pharmacia PhastSystem was used for isoelectric point determination following the procedure prescribed by the manufacturer of the PhastSystem. Precast PhastGel IEF gels were used with a pH range of 3 to 9. Gels were stained for proteins or peptides either with 0.02% of Coomassie R-250 (Pharmacia, Sweden) as directed in the PhastGel procedure for IEF or silver staining using a Bio-Rad kit following the procedure prescribed by the manufacturer. Pharmacia broad pI calibration kit (pH 3-10) was used as standards in isoelectric focusing.

Isoelectric focussing was also carried out using LKB Ampholine PAGplates (5x11 cm) with a pH gradient of 3 to 9.5 on Pharmacia flat-bed electrophoresis apparatus, with a computer-controlled electrophoresis power supply (Bio-Rad Model 3000Xi). Separation was carried out at 200 V for 10 min for prefocussing and 350 V for 30 min for separation. The method of the manufacturer was modified to extend the pH gradient to 11.5 to suit the individual experiment. The modification was involved putting a saturated filtered paper with ethanolamine across the cathodal end of the gel and run the apparatus for 30 minutes to create a new gradient. A JULABO F10 cooler was used to keep the temperature at 15°C.

The isoelectric focussing gels were stained with Coomassie Blue R 250 following the procedure given in the booklets supplied with the gels or by silver staining using a Bio-Rad kit following the procedure prescribed by the manufacturer.

2.16 Radioiodination

Radioiodination of proteins and peptides was achieved by using a solid-phase oxidising agent (Iodogen) in the method of Salacinski *et al.* (1981) as modified by Hodgkinson *et al.* (1987).

2.16.1 Preparation of iodogenated vial

200 µl of Iodogen (1 mg per 12.5 ml of dichloromethane) was measured into a 0.75 ml Eppendorf vial. The vial was placed uncapped in a drawer overnight so the dichloromethane could evaporate, leaving a coating of oxidant on the inner walls of the vial.

2.16.2 Iodination reaction

10 µl of peptide or protein solution (100 µg of protein or peptide) was placed into an iodogenated vial. To this 20 µl of 0.064 mol/l phosphate buffer pH 7.4 was added. Then a 5 µl (0.5 mCi) of ^{125}I iodine (Amersham International plc, Amersham, Bucks, U.K.) was added and the contents of the tube were mixed. The iodination was allowed to proceed for 10 min then the tube contents were transferred to a non-iodogenated vial containing 50 µl of 0.064 mol/l phosphate buffer pH 7.4. Unincorporated ^{125}I iodine was separated from the labelled peptides by passing the material through a SepPak cartridge for solid-phase extraction (Waters Associates, Milford, MA, U.S.A) as described by Hodgkinson *et al.* (1987).

The ^{125}I-labelled peptide was stored at -20°C and used within 24 h.

2.17 Amino acid analysis

A concentrated salt-free sample of the protein or peptide was taken for amino acid analysis. Amino acid analysis of the samples were kindly carried out by Mr C. Monk of the University of Waikato, Hamilton. The samples were freeze-dried, then hydrolysed in 6 mol/l HCl at 110°C for 24 h under vacuum and the amino acids were separated as phenylthiocarbamyl derivatives on reversed phase HPLC. .

The separation was carried out following the procedure in the Waters PICO-749 amino acid analysis system operator's manual as described by Bidlingmeyer *et al.* (1984).

2.18 Ultrafiltration

Ultrafiltration of material was by means of a stirred cell pressurized with nitrogen gas. The ultrafiltration apparatus was manufactured by Amicon Corp. (Lexington, Mass., U.S.A).

2.19 Rotary evaporation

Rotary evaporation of samples was carried out under vacuum at a temperature of 35 to 40°C using a Buchi Rotovapor.

2.20 Statistical analysis

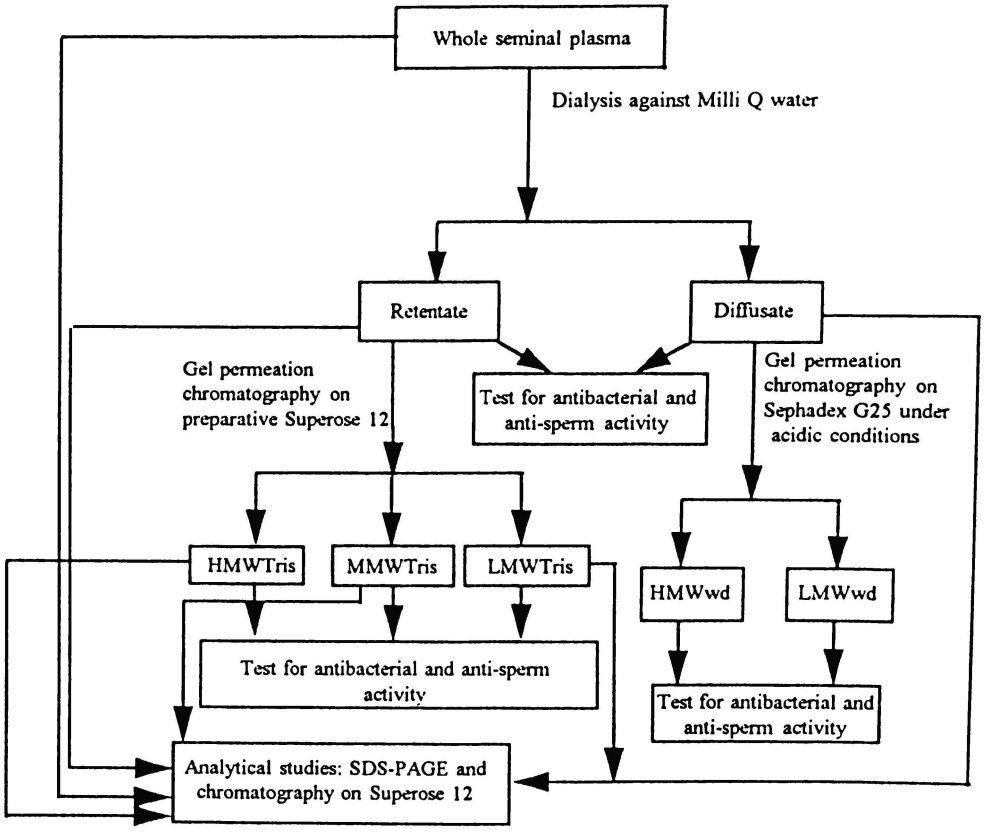
Analysis of variance was as outlined by Snedcor and Cochran (1972). Significant differences between treatment means were determined by Duncan's multiple range test.

2.21 Reagents

Acetonitrile, B.D.H. HiPerSolv
Ammonium sulphate, Peking Chemical Works AR grade.
Bovine serum albumin, SIGMA
Bromophenol blue, Koch-Light AR
Caproic acid, B.D.H. LR
Chloromycetin, Park-Davis
Citric acid, M&B. AR
Di-and tri-sodium citrate, M&B. AR
Dimethyl sulphoxide
Ficoll 400, Pharmacia
Glycerol, B.D.H. LR
Glycine, B.D.H. AR
Hydrochloric acid, BDH, HiPerSolve, HPLC grade.
β-Mercaptoethanol, B.D.H. Biochemical
Penicillin, Glaxo
Propan-2-ol (isopropanol)), HiPer Solv BDH.
Sodium dodecyl sulphate (SDS), SIGMA
Sodium hydroxide, BDH, LR grade.
Sodium nitroprusside, Univar AR grade.
Sodium sulphacetamide, B.D.H LR
Streptomycin, Glaxo
Tetrabutylammonium hydrogen sulphate for HPLC, BDH HiPer Solv.
Trifluoroactic acid, Pierce HPLC grade.
Tris (hydroxymethyl) aminomethane , SIGMA.

Water (general), Milli Q
Water for reverse phase chromatography, Milli Q filtered through a Millipore Norganic Cartridge.

Flow chart for Sections 3.1 and 3.2



CHAPTER THREE
PRELIMINARY STUDIES TO IDENTIFY THE ANTI-SPERM ACTIVITY IN BOVINE SEMINAL PLASMA

3.1 PRELIMINARY STUDY

As detailed in Chapter One, the effect of bovine seminal plasma on sperm survival has been extensively studied by numerous researchers. However, the function of many proteins and peptides in seminal plasma is not sufficiently understood. Various fractions from seminal plasma have been found to affect sperm motility and both high and low molecular weight proteins and peptides in bovine seminal plasma have been implicated in the anti-sperm activity.

Bovine seminal plasma contains low molecular weight proteins and peptides which have been reported to pass through a dialysis membrane with a 3.5 kDa cut-off (Baas, 1985), and through an ultrafilter of 2 kDa retentivity at pH 3, and at pH 7 in the presence of citrate, but not without it (Shannon *et al.*, 1987). These low molecular weight peptides aggregated to a molecular weight of 30 kDa at neutral pH (Shannon *et al.*, 1987; Hameed, 1987). It was aimed to investigate more thoroughly these low molecular weight components in their native state and study their anti-sperm activity.

3.1.1 DIALYSIS OF SEMINAL PLASMA

Dialysis was conducted at neutral pH to separate the low molecular weight components of seminal plasma from the high molecular weight components in their native state.

Accordingly, 10 ml of fresh bovine seminal plasma obtained from pooled fresh ejaculates of three bulls was placed in a dialysis membrane with a 12 kDa cut-off and dialysed for 48 hours at 4°C against two litres of Milli Q water. After 48 hours the retentate appeared as a milk-like solution with heavy precipitation. This indicated a change in the solubility of the proteins in the seminal plasma after dialysis. The volume of the diffusate and of the retentate was reduced to 10 ml by rotary evaporation before further processing.

The precipitated material in the retentate could be re-dissolved by the addition to the retentate sodium citrate (to 0.1 mol/l, pH 6.9), or Tris (to 0.1 mol/l, pH 7), or NaCl (to 0.9%); however, the solution still appeared to be slightly cloudy.

A primary screening for the antibacterial and anti-sperm activity in the fractions of seminal plasma was performed as the resulting material from the dialysis was used for further investigation.

3.1.2 SCREENING FOR ANTIBACTERIAL ACTIVITY

It was suggested by Shannon *et al.* (1974) that the anti-sperm and antibacterial activity in bovine seminal plasma may be due to the same components. They found both anti-sperm and antibacterial activity in all fractions obtained by differential acetone precipitation of bovine seminal plasma. It is not clear from this report whether the same components are responsible for both anti-sperm and antibacterial activities. Also, the biochemical nature of these components was not described.

Shannon *et al.* (1987) found that the antibacterial activity in bovine seminal plasma was reduced after dialysis against water and the diffusate was reported to have trace antibacterial activity (Shannon *et al.*, 1974; 1987). Seminalplasmin, a cationic protein of molecular weight of 5.4 kDa with antibacterial and anti-sperm properties (Shivaji, 1988) was expected to be present in the diffusate. An antibacterial assay was conducted to determine how much of the activity had passed through the dialysis membrane and how much of the activity was retained.

Accordingly, aliquots of 100 µl of whole seminal plasma, the retentate of dialysed seminal plasma, and the diffusate of dialysed seminal plasma were tested for antibacterial activity by using the well diffusion technique on agar plates. The results in Table 1 show that both the retentate and the diffusate of seminal plasma have antibacterial activity. However, most of the activity was retained on dialysis.

Table 1. Antibacterial activity of whole seminal plasma, the diffusate and the retentate of dialysed seminal plasma (tested against *E. coli*).
Values are mean ± s.e.m (n=3)

Sample	Mean Clearance Zone (mm^2)
Whole seminal plasma	69.7 ± 5.7^a
The retentate of dialysed seminal plasma	59.7 ± 10.7^a
Diffusate	23.75 ± 4.2^b

Like superscripts within a column indicate no significant difference between means ($p<0.05$)

3.1.3 SCREENING FOR ANTI-SPERM ACTIVITY

To determine whether the fractions have anti-sperm activity similar to the antibacterial activity, a test on washed bull sperm was conducted.

3.1.3.1 PREPARATION OF SAMPLES

A 1 ml sample of the retentate obtained from dialysis of seminal plasma was rotary evaporated to dryness and the residue was dissolved in 1 ml of 14G diluent. The solution was centrifuged for 10 min at 20 000 *g*, and the light sediment of material which did not dissolve in 14G buffer was separated from the supernatant. The supernatant was designated DSP_{14G}. A few drops of dimethyl sulphoxide (DMSO) were added to the sediment in an attempt to redissolve the precipitate, which was then resuspended in 1 ml of 14G diluent. The solution appeared clear and it was designated DSP_{DMSO}.

3.1.3.2 TESTING FOR ANTI-SPERM ACTIVITY

The DSP_{14G} and DSP_{DMSO} fractions, a control of few drops of dimethyl sulphoxide in 1 ml of 14G, the diffusate and whole seminal plasma were tested for their effects on sperm motility. A 100 µl sample of each of these was added to 900 µl of a suspension of washed sperm in 14G diluent containing 0.1% bovine serum albumin, to give a 10% concentration of each sample and a sperm concentration of $10\text{-}12\text{x}10^6$ sperm/ml. A control with no seminal plasma or fractions added was included (*i.e.* 100 µl of 14G diluent was added to the 900 µl of sperm suspension). The mixture for each treatment was incubated for 18 h at ambient temperature. Visual assessment of motility was conducted immediately after the additions (considered to be time zero) and after 18 h incubation.

The results (in Table 2) indicated that seminal plasma had a detrimental effect on sperm motility. The percentage of motile sperm after 18 h was higher in the control (*i.e.* where seminal plasma or its fractions were not added). The addition of whole seminal plasma, the retentate of dialysed seminal plasma, the precipitated material which dissolved in dimethyl sulphoxide and the diffusate all reduced the motility of sperm. The extent of the effect was different, however. While whole seminal plasma and all fractions from seminal plasma had an adverse effect on the motility of sperm after 18 h incubation, the material which did not dissolve in the buffer and required dimethyl sulphoxide for dissolving exerted the highest anti-sperm activity. Dimethyl sulphoxide appeared to have no effect on the motility of the sperm.

Table 2. Effect of the diffusate and fractions of the retentate of dialysed seminal plasma on the motility of washed bull sperm

The values for motility are the percentage of motile sperm immediately after addition and after 18 h incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. mg/ml	% Motile	
		0 h	18 h
Control (diluent)		83.3±1.7[a]	71.7±1.8[a]
DSP_{14G}	3.63	83.3±1.7[a]	63.4±4.4[b]
DSP_{DMSO}	0.23	71.7±1.8[b]	38.5±3.3[c]
Diffusate	0.20	83.3±1.7[a]	63.3±4.4[b]
Dimethyl sulphoxide		83.3±1.7[a]	73.3±1.7[a]
Whole seminal plasma	4.2	81.7±1.8[a]	65.0±2.9[b]

Like superscripts within a column indicate no significant difference between means ($p<0.05$)

Overall, there was a marked increase in the anti-sperm activity. There was as much activity in the diffusate as there was in the original seminal plasma, and much more in the retentate. It seems that dialysis of seminal plasma caused an increase in anti-sperm activity.

It was thought that bovine serum albumin might have some sperm-protecting property, or that it might reduce the detrimental effects of the anti-sperm components. Thus, the experiment was repeated with an increase in the bovine serum albumin concentration to 1%. The result was the same and there was no reversal of the damaging activity.

3.1.4 SUMMARY AND CONCLUSION:

This initial study confirms that components in seminal plasma have antibacterial and anti-sperm activity. The activity was present in both high and low molecular weight components (*i.e.* the retentate and the diffusate). Although antibacterial and anti-sperm activity passed through the dialysis membrane, the retentate of dialysed seminal plasma retained high antibacterial and anti-sperm activity.

There was an increase in the total anti-sperm activity after dialysis. The retentate and the diffusate of seminal plasma exerted anti-sperm activity as much as the seminal plasma, and the material dissolved in dimethyl sulphoxide had higher activity.

3.2 PRELIMINARY IDENTIFICATION OF THE PROTEINS OF VARIOUS MOLECULAR WEIGHTS IN BOVINE SEMINAL PLASMA WITH ANTI-SPERM ACTIVITY

The preliminary study demonstrated that dialysed seminal plasma had an adverse effect on sperm motility. It was also shown that the antibacterial and anti-sperm activity did not all pass through the dialysis membrane of 12 kDa molecular weight cut-off. It was decided to further investigate the effect of the various components in the retentate of dialysed seminal plasma individually to determine where the anti-sperm activity resides and whether the separation of the various components affect the anti-sperm activity *per se*.

3.2.1. PREPARATIVE GEL PERMEATION CHROMATOGRAPHY OF THE RETENTATE OF DIALYSED SEMINAL PLASMA

Proteins in bovine seminal plasma under conditions of neutral pH are reported to exist in two major aggregates, *i.e.* 500 kDa and 250 kDa and a small amount of a 20 kDa aggregate (Hameed, 1987). It has previously been shown by Hameed (1987) that the antibacterial activity exists in the form of aggregates. The aim of this experiment was to separate these various fractions in seminal plasma by chromatography under non-disaggregating conditions and test their effect on sperm motility.

Accordingly, to 5 ml of the retentate of dialysed seminal plasma, an equal volume of 0.2 mol/l Tris-HCl, pH 7 was added. This solution was centrifuged at 20 000 *g* for 10 minutes. A 2 ml sample of the clear supernatant was then injected onto a preparative Superose 12 column and chromatographed as detailed in Fig. 1. The elution profile in Fig. 1 shows several peaks of various molecular weights. The eluted proteins covered a wide range of molecular weights between 300 and 10 kDa.

The chromatography was repeated four times, where identical elution profiles were obtained, so that sufficient material was generated to test for anti-sperm and antibacterial activity.

3.2.1.1 SCREENING FOR ANTI-SPERM AND ANTIBACTERIAL ACTIVITY IN THE FRACTIONS IN FIG. 1

Primary screening of the fractions shown in Fig. 1 for antibacterial and anti-sperm activity was conducted to determine in which molecular weight region the two activities mostly reside, as it was reported by Shannon *et al.* (1974) that the anti-sperm and the antibacterial activity may be due to the same components. This screening for the activity in the fractions obtained from the chromatography of

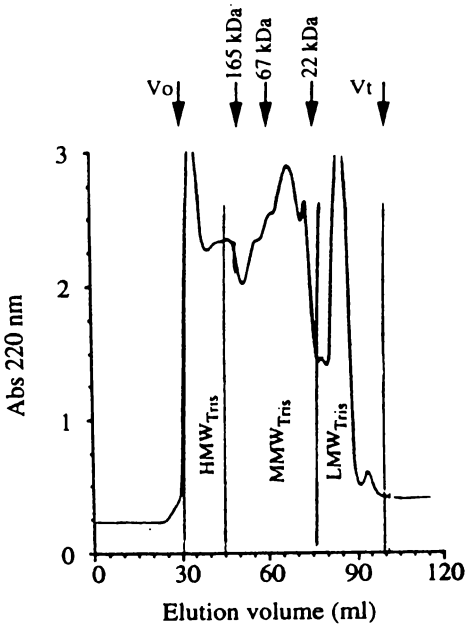


Fig. 1: Gel permeation chromatography of the retentate of dialysed seminal plasma.
Column: Preparative Superose 12.
Sample: 2 ml of the mixture of the retentate of dialysed seminal plasma 1:1 with 0.2 mol/l Tris-HCl, pH 7.0
Eluent: 0.1 mol/l Tris-HCl buffer, at pH 7.0.
Flow rate: 1 ml/min.

seminal plasma would determine if the activities are confined to the same component(s).

Preparation of samples for preliminary assessment of antibacterial and anti-sperm activity

For the purposes of primary screening for antibacterial and anti-sperm activity, the material eluted from the Superose 12 column was collected in 5 ml fractions which were pooled from the repeated runs into three molecular weight groups:

- high molecular weight group composed of fractions 6 to 9 and designated HMW_{Tris},
- medium molecular weight group comprising fractions 10 to 15 designated MMW_{Tris},
- relatively low molecular weight group comprising fractions 16 to 20 designated LMW_{Tris}.

The pooled fractions were reduced in volume by rotary evaporation to 5 ml (the original volume of seminal plasma injected onto the column). The concentrated fractions were then each dialysed against 2 litres of Milli Q water using dialysis tubing with molecular weight cut-off of 1 kDa to remove the buffer salt. The dialysis was carried out for a period of 48 hours to ensure that the salt had sufficient time to diffuse out of the membrane. After the dialysis was completed, the volume of each retentate was reduced to 5 ml by rotary evaporation. The protein concentration of the retentate of each group in Fig. 1 was determined and it was:

HMW_{Tris}	11.2 mg/ml
MMW_{Tris}	17.2 mg/ml
LMW_{Tris}	6.3 mg/ml

The volume of the retentates was then reduced by rotary evaporation to 2.5 ml (half the original volume of the seminal plasma that was chromatographed) and 1 ml sample of each was then diluted 1:1 with DS14G diluent to be tested for anti-sperm activity.

Test for antibacterial activity

A 0.5 ml sample of each of the concentrated retentate of the fractions shown in Fig. 1 was diluted with 0.2 mol/l sodium citrate at pH 6.9. Antibacterial activity was determined and the results are shown in Table 3. The antibacterial activity was spread over a wide range, however it was significantly higher in the medium and high molecular weight fractions than the low molecular weight fractions.

Table 3. Antibacterial activity of whole and dialysed bovine seminal plasma and the HMW_{Tris}, MMW_{Tris} and LMW_{Tris} fractions (tested against *E. coli*).
Values are mean ± s.e.m (n=3)

Sample	Mean Clearance Zone (mm^2)
HMW_{Tris}	53.5 ± 2.4^{c}
MMW_{Tris}	51.4 ± 2.4^{c}
LMW_{Tris}	32 ± 2.0^{d}
DSP*	61.4 ± 2.6^{b}
Whole seminal plasma	78.1 ± 2.9^{a}

**Retentate of dialysed seminal plasma*

Like superscripts within a column indicate no significant difference between means ($p<0.05$)

Test for anti-sperm activity

From each of the prepared samples mentioned above, 100 µl was added to 900 µl of a suspension of washed sperm in 14G diluent containing 0.1% bovine serum albumin to give 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. Controls with 100 µl of the retentate of dialysed seminal plasma and 100 µl of 14G diluent instead of seminal plasma or added fractions was included. The mixture for each treatment was incubated for 18 h at ambient temperature. Visual assessment of motility was conducted immediately after addition (considered to be time zero) and after 18 h incubation. The results are shown in Table 4.

Table 4. Effect of the fractions of seminal plasma from Fig 1 on the motility of washed bull sperm.
The values for motility are the percentage of motile sperm immediately after addition and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		83.3 ± 1.7^{a}	71.7 ± 1.7^{a}
HMW_{Tris}	1.12	81.7 ± 1.8^{a}	58.3 ± 4.4^{b}
MMW_{Tris}	1.72	83.3 ± 1.7^{a}	73.3 ± 1.7^{a}
LMW_{Tris}	0.63	75.0 ± 2.9^{b}	40.0 ± 2.9^{c}
DSP*	3.63	80.0 ± 0.0^{a}	65.0 ± 2.9^{b}

Retentate of seminal plasma dialysed against water at 10% concentration for comparison.
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The results (in Table 4) clearly show that the HMW_{Tris} and LMW_{Tris} fractions had an adverse effect on the motility of sperm after 18 h incubation. The reduction in the percentage of motile sperm was significant. The fraction of relatively low molecular weight (LMW_{Tris}), however, possessed the highest anti-sperm activity, although it was lowest in protein concentration. It is also evident that the HMW_{Tris} had more adverse effect on motility than the MMW_{Tris} which had the highest protein concentration.

3.2.1.2 SUMMARY AND CONCLUSION:

From the results obtained in this experiment, there is evidence that the proteins in the retentate of dialysed seminal plasma are of a wide range of molecular weights. These components have antibacterial and anti-sperm activity.

The antibacterial activity, however, was higher in the high and the medium molecular weight region than the low molecular weight region, while the anti-sperm activity was higher in the low molecular weight region than the high molecular weight region. It is noteworthy that the medium molecular weight region has no anti-sperm activity despite having the highest protein concentration and a high antibacterial activity.

Previous workers reported that the anti-sperm activity in seminal plasma is confined to the high molecular weight cationic components (Baas, 1985). However, recent work has described the anti-sperm activity as being higher in low molecular weight fractions of less than 20 kDa (Peitz, 1988; Collingwood, 1990). This was confirmed by the preliminary results in the present study. The nature of the anti-sperm activity in the bovine seminal plasma was further investigated.

3.2.2 ANALYTICAL STUDY OF THE COMPONENTS IN SEMINAL PLASMA

3.2.2.1. ANALYTICAL GEL PERMEATION CHROMATOGRAPHY

The result in Fig.1 indicated that the proteins in bovine seminal plasma exist in a wide range of molecular weights, but were not well resolved by the preparative chromatography column. Thus, in order to resolve the fractions comprising each area, the retentate of dialysed seminal plasma was chromatographed on an analytical Superose 12 column.

Accordingly, 50 µl of the retentate of dialysed seminal plasma mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7.0 was injected onto the analytical Superose 12 column. The

details and the elution profile are shown in Fig. 2. A better resolution of the components in seminal plasma was achieved (5 distinct peaks) with the analytical column than with the preparative column. Each of the peaks in Fig. 2, however, may represent more than one component.

3.2.2.2 SDS-PAGE OF BOVINE SEMINAL PLASMA

The profile of the chromatography of the retentate of dialysed seminal plasma showed several peaks of various molecular weights. To determine the composition and the molecular weights of the protein components in seminal plasma, the retentate of dialysed seminal plasma was subjected to SDS-PAGE on 12.5% polyacrylamide gel under reducing and non-reducing conditions. Whole seminal plasma was included for comparison. The results (in Fig. 3) show more than 10 bands with molecular weights ranging between 200 and 14 kDa. The major band, however, both under reducing and non-reducing conditions had an estimated molecular weight of 16-18 kDa. It is noteworthy that all samples showed a smear below the band of 14 kDa. While the chromatography profile under neutral conditions indicated that the majority of the proteins in bovine seminal plasma are of high molecular weights (>20 kDa), the results from SDS-PAGE suggest that this is due to a large part to aggregation of proteins of relatively low molecular weights (<20 kDa).

This shift in molecular weight could be due to the effects of SDS on the aggregates causing disaggregation of the higher molecular weight components to the relatively low molecular weight. Kemme *et al.* (1986) reported that the major protein in bovine seminal plasma appeared in the region of 14 to 16 kDa upon SDS-PAGE under reducing conditions. However, no explanation for the disaggregation was given in their report and there was no indication of the native state of the aggregates.

3.2.2.3 RE-CHROMATOGRAPHY OF THE FRACTION OF HIGH MOLECULAR WEIGHT IN FIG. 1

To test the hypothesis that the proteins in seminal plasma exist as high molecular weight aggregates in equilibrium with the relatively low molecular weight components, a sample from the void volume peak from the chromatography of seminal plasma on the preparative Superose 12 column (HMW_{Tris} in Fig. 1) was incubated overnight at 4°C in the elution buffer (0.1 mol/l Tris-HCl, pH 7). A 100 µl aliquot was then chromatographed on the Superose 12 analytical column equilibrated with the same buffer. The elution profile (shown in Fig. 4) indicated the presence of components of lower molecular weight estimated to be between 10 and 40 kDa. Incubation overnight at 4°C was to test the hypothesis that the protein aggregates of

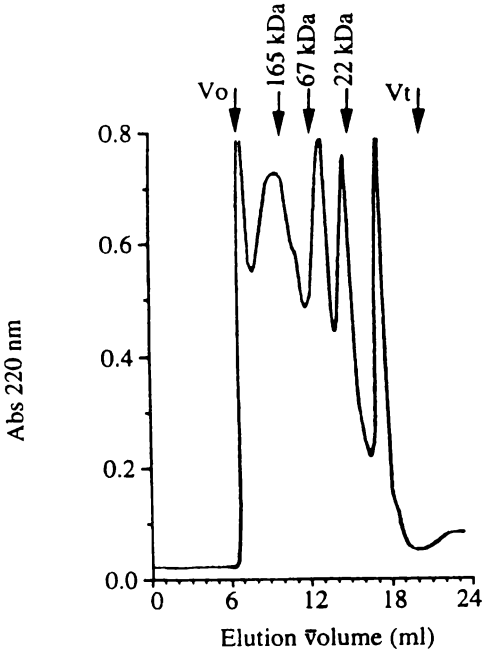


Fig. 2: Gel permeation chromatography of the retentate of dialysed seminal plasma.
Column: analytical Superose 12 HR10/30.
Sample: 50 µl of the retentate of dialysed seminal plasma mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7.0
Eluent: 0.1 mol/l Tris-HCl buffer, at pH 7.0.
Flow rate: 0.5 ml/min.

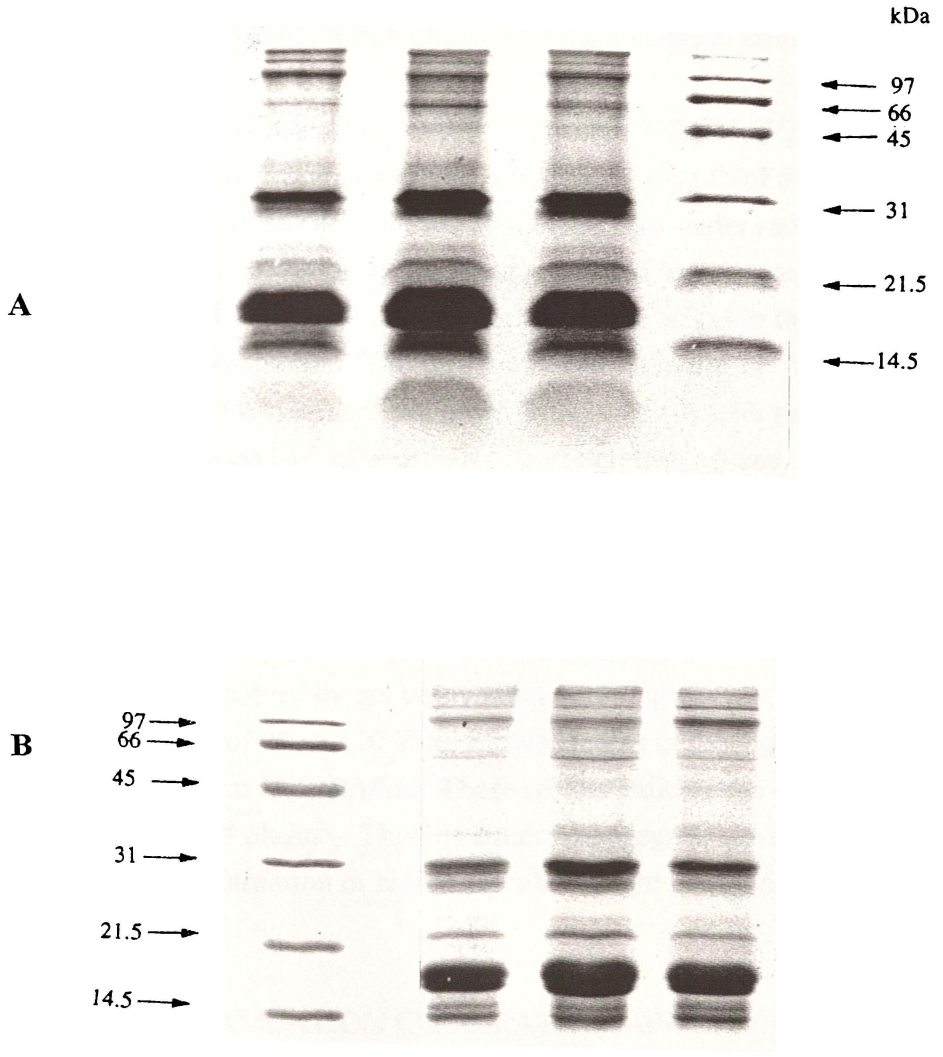


Fig. 3:. SDS-PAGE of seminal plasma under reducing and non-reducing conditions in the presence of SDS. 10 µl sample was loaded on a 12.5% polyacrylamide gel.

A- Under non-reducing conditions. Lane 1: the retentate of dialysed seminal plasma. Lane 2 and 3: whole seminal plasma. Lane 4: molecular weight markers (97 to 14.5 kDa).

B- Under reducing conditions. Lane 1: molecular weight markers (97 to 14.5 kDa). Lane 2: the retentate of dialysed seminal plasma. Lane 3 and 4: whole seminal plasma.

high molecular weight exist in equilibrium with the low molecular weight aggregates. However, disaggregation was not complete. The cold temperature and dilution may have affected hydrophobic aggregation and favoured the release of some of the lower molecular weight components associated with the larger components.

3.2.2.4 SDS-PAGE OF THE THREE FRACTIONS IN FIG. 1

In an attempt to further explore the composition of each of the groups in Fig. 1, it was decided to subject the fractions to SDS-PAGE under reducing conditions. Accordingly, a 10 µl sample of each was prepared for electrophoresis as described in Chapter Two and loaded on a 12.5% polyacrylamide gel in the presence of SDS under reducing conditions. The results are shown in Fig. 5. While both HMW_{Tris} and MMW_{Tris} were resolved into more than 10 bands with molecular weights ranging between 200 kDa and 14 kDa, a major band between 16 and 18 kDa was apparent in all fractions. Other bands in the high molecular weight region were also common to the three fractions. It is interesting that in the low molecular weight fraction (LMW_{Tris}) protein bands were present within a region of higher molecular weight than that estimated by gel permeation chromatography. The medium molecular weight fraction resolved by gel permeation chromatography, corresponding to molecular weight of 20 to 150 kDa, showed bands in the region of molecular weight less than 14 kDa on SDS-PAGE. These results indicate the complexity of the proteins in seminal plasma. There is either reaggregation even in the presence of SDS, or major retardation of high molecular weight components by interaction with Superose 12.

3.2.2.5 GEL PERMEATION CHROMATOGRAPHY OF THE PRECIPITATED MATERIAL FROM THE RETENTATE OF DIALYSED SEMINAL PLASMA

Material that precipitated from the retentate on dialysis of seminal plasma and did not redissolve in the citrate buffer, but partially dissolved when dimethyl sulphoxide was added, was seen to exert the highest anti-sperm activity. To investigate its protein composition, this material was chromatographed on the analytical Superose 12 column. The precipitate from 1 ml of seminal plasma was treated with a few drops of dimethyl sulphoxide and resuspended in 0.1 mol/l Tris-HCl buffer at pH 7. It was then centrifuged at 20 000 *g* for 10 min, and 200 µl of the clear supernatant was injected onto the analytical Superose 12 column eluted with 0.1 mol/l Tris-HCl buffer at pH 7. The results are shown in Fig. 6. The elution profile shows several peaks of various molecular weights, however, the bulk of the protein was eluted at the relatively low molecular weight region with an estimated molecular weight of 20 kDa. It is clear from the chromatography that the material dissolved in dimethyl sulphoxide

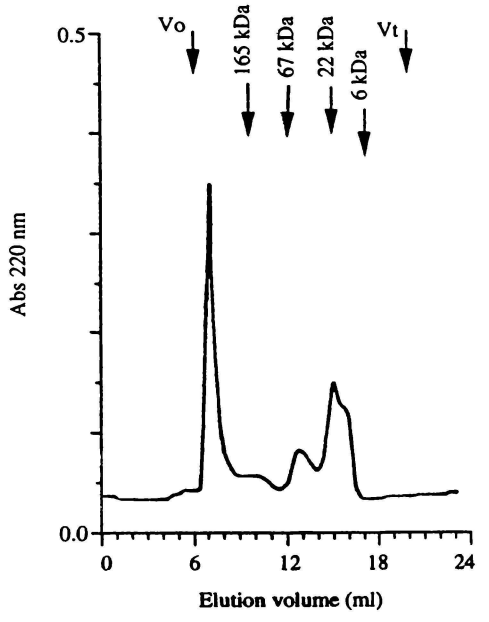


Fig. 4: Gel permeation chromatography of void volume peak in Fig. 1.
Column: analytical Superose 12 HR10/30.
Sample: 100 µl of the sample incubated overnight in 0.1 mol/l Tris-HCl, pH 7.0
Eluent: 0.1 mol/l Tris- HCl buffer, pH 7.0
Flow rate: 0.5 ml/min.

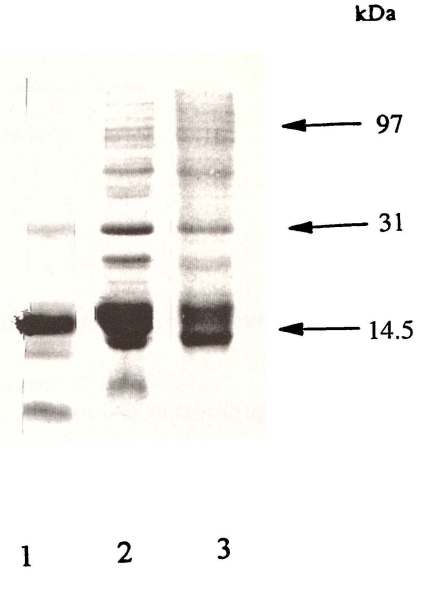


Fig. 5: SDS-PAGE under reducing conditions of the fractions in Fig. 1. A 10 µl sample was loaded on a 12.5% polyacrylamide gel.
Lane 1: LMW_{Tris}. Lane 2: MMW_{Tris}. Lane 3: HMW_{Tris}.

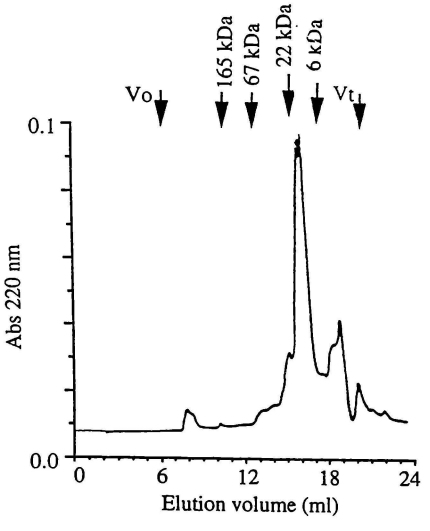


Fig. 6

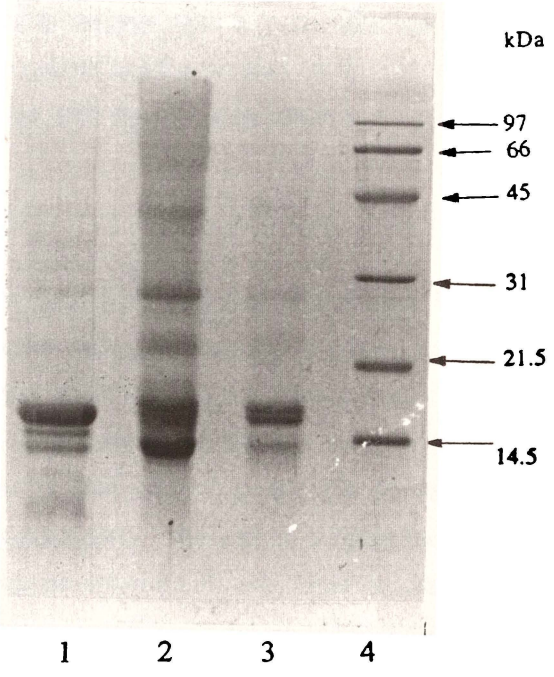


Fig. 7

Fig. 6: Gel permeation chromatography of precipitated material from the retentate of dialysed seminal plasma.
Column: analytical Superose 12 HR10/30.
Sample: 200 µl of the precipitate dissolved in dimethyl sulphoxide and 0.1 mol/l Tris-HCl, at pH 7.0
Eluent: 0.1 mol/l Tris- HCl buffer, pH 7.0.
Flow rate: 0.5 ml/min

Fig. 7: SDS-PAGE under reducing conditions of the precipitated material from the retentate of dialysed seminal plasma which was dissolved by the addition of dimethyl sulphoxide.
Lane 1: the precipitated material. Lane 2: bovine seminal plasma. Lane 3: LMW_{Tris}. Lane 4: molecular weight markers.

from the precipitate of seminal plasma comprised mostly of components of relatively low molecular weight less than 40 kDa.

3.2.2.6 SDS-PAGE OF THE PRECIPITATED MATERIAL FROM THE RETENTATE OF DIALYSED SEMINAL PLASMA

In order to determine the molecular weight of the components in the precipitate, the clear supernatant mentioned above was subjected to SDS-PAGE under reducing conditions. Accordingly, a 10 µl sample was mixed 1:1 with 5 times concentrated sample buffer to allow a less diluted sample, and 10 µl from this was loaded on a 12.5% polyacrylamide gel. A 10 µl samples from seminal plasma and the low molecular weight fractions in Fig. 1 were loaded for comparison. The results (in Fig. 7) show that the components in the precipitated material that dissolved in dimethyl sulphoxide were mainly of relatively low molecular weight with a trace of some bands at the high molecular weight area. As before, a major band common to the three samples was observed in the region 16-18 kDa as well as minor bands in the higher molecular weight region.

3.2.2.7 SUMMARY AND CONCLUSION:

From these preliminary results it appears that the proteins in seminal plasma are in an aggregated form. Gel permeation chromatography showed a variety of molecular weights ranging between 10 and 300 kDa. SDS-PAGE of seminal plasma under reducing and non-reducing conditions revealed the presence of more than 10 protein bands. The reducing condition did not affect the profile indicating that disulphide bonding does not appear to contribute to the aggregation. The major protein band was in the relatively low molecular weight region of between 16 and 18 kDa. In contrast to this, seminal plasma protein on chromatography at neutral pH appeared to exist as high molecular weights aggregates. SDS-PAGE of the various fractions in Fig. 1 revealed the complexity of these fractions and once again all fractions have a common bands at the region between 14 and 18 kDa. Interestingly, the fractions of estimated molecular weigh by gel permeation chromatography of less than 20 kDa showed a trace of bands at the high molecular weight region. The incubation overnight of the high molecular weight components at 4°C had an effect on the status of the aggregates possibly due to disaggregation or dilution effects: re-chromatography of the incubated material on analytical Superose 12 revealed the presence of components in the lower molecular weight region with estimated molecular weights of 10 to 40 kDa.

3.2.3 A STUDY OF THE DIFFUSATE FROM THE DIALYSIS OF SEMINAL PLASMA AGAINST WATER

The diffusate of seminal plasma dialysed against water exhibited anti-sperm as well as antibacterial activity. It was decided to further investigate the nature of this material since there have been no previous reports on the anti-sperm activity of the diffusate.

3.2.3.1 SDS-PAGE OF THE DIFFUSATE FROM THE DIALYSIS OF SEMINAL PLASMA AGAINST WATER

The material that appeared in the diffusate was presumably of molecular weight of less than 12 kDa (*i.e.* the retention capacity of the dialysis membrane). To determine the molecular weight of the proteins or peptides contained in the diffusate of seminal plasma dialysed against water it was subjected to SDS-PAGE under reducing conditions. Due to the expected low molecular weight of these components a 20% polyacrylamide gel was chosen and the electrophoresis was performed as described in Chapter Two. A 10 µl sample of the concentrated diffusate was mixed 1:1 with 5 times concentrated sample buffer to allow a less diluted sample to be loaded onto the gel. The results (see Fig. 8) showed that the diffusate contains components that are proteinaceous in nature. The two main bands detected had estimated molecular weights of about 8 kDa and 3 kDa and a faint band was observed at the lower molecular weight region of around 2 kDa.

3.2.3.2 GEL PERMEATION CHROMATOGRAPHY OF THE WATER DIFFUSATE ON THE SUPEROSE 12 COLUMN

To determine the composition of the water diffusate and its aggregation capacity, gel permeation chromatography under neutral conditions was performed. Accordingly, 500 µl of the concentrated diffusate was mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7 and incubated for 2 h at 37°C. The mixture was then chromatographed on an analytical Superose 12 column and eluted with 0.1 mol/l of Tris-HCl, pH 7 as detailed in Fig. 9. The elution profile (in Fig. 9) shows that the diffusate is comprised of components with molecular weights less than 20 kDa. However, since these relatively low molecular weight components were poorly resolved, a further separation was conducted.

3.2.3.3 GEL PERMEATION CHROMATOGRAPHY OF THE DIFFUSATE RESULTING FROM THE DIALYSIS OF BOVINE SEMINAL PLASMA AGAINST WATER ON SEPHADEX G25

As reviewed in Chapter One, the diffusate of seminal plasma dialysed against water is known to have a beneficial effect on sperm motility. It was found that the material

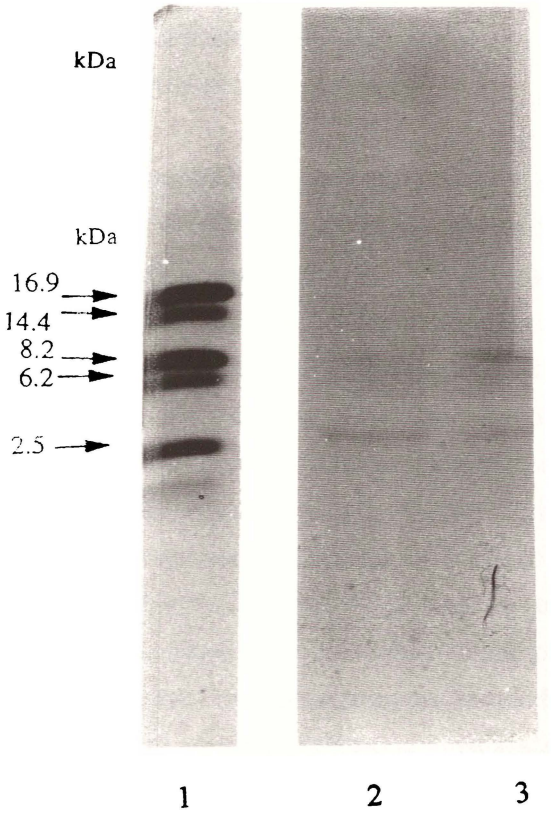


Fig. 8

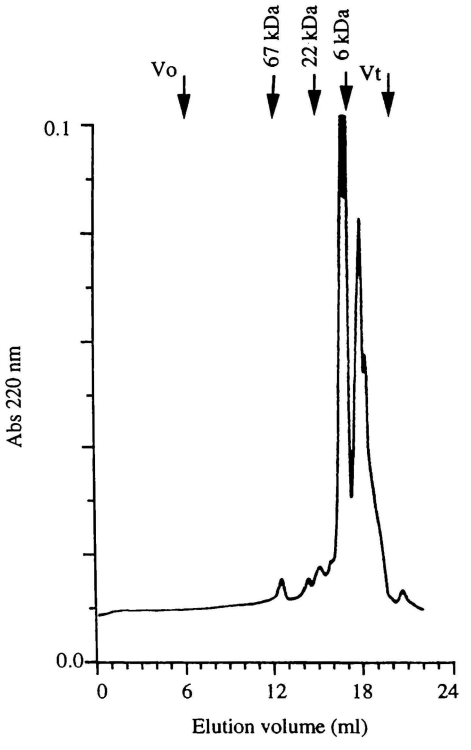


Fig. 9

Fig. 8: SDS-PAGE under reducing conditions of the water diffusate on a 20% polyacrylamide gel.
Lane 1: molecular weight markers. Lane 2 and 3: water diffusate.

Fig. 9: Gel permeation chromatography of the water diffusate
Column: analytical Superose 12 HR10/30.
Sample: 500 µl of the concentrated water diffusate mixed 1:1 with 0.2 mol/l Tris -HCl, pH 7.0
Eluent: 0.1 mol/l Tris- HCl buffer, pH 7.0
Flow rate: 0.5 ml/min

that passed through a 500 dalton cut-off ultrafilter stimulated the motility of washed bull sperm, and that motility was sustained for a longer period than in whole seminal plasma as well as in the retentate of dialysed seminal plasma (Baas *et al.*, 1983). The ultrafilter retentate was not tested for anti-sperm activity. Shannon *et al.* (1987) found that the antibacterial activity of seminal plasma was reduced when seminal plasma was dialysed against water. Shannon (personal communication) suggested that the beneficial effect in the whole diffusate is probably due to the domination of the beneficial component(s).

In an attempt to separate the components in the diffusate with molecular weights of less than 5 kDa, the diffusate prepared as described in Section 3.1.1 was concentrated to 4.5 ml by rotary evaporation and the pH was adjusted to 1.7 with HCl. The sample was then chromatographed on a Sephadex G25 column eluted with 0.02 mol/l HCl. Low pH was used to suppress the ionic attraction between the molecules in an attempt to dissociate them. The elution profile (in Fig. 10) showed a peak at the void volume followed by a large peak with a molecular weight less than 5 kDa (the exclusion size of Sephadex G25). This second peak may have contained small peptides as well as mineral ions, amino acids, and citrate. The absorbance maximum of citrate is at 220 nm which may be responsible for the size of the peak. The fractions comprising the void volume peak in Fig. 10 were pooled, the pH was adjusted to 7 and their volume reduced to 5 ml (half the original volume of the sample of seminal plasma dialysed against water). The fractions comprising the second peak were treated in similar manner. The protein concentration of the pooled concentrated fractions was determined using the Biuret method. A 0.5 ml sample of each was mixed 1:1 with 0.2 mol/l sodium citrate at pH 6.9 for testing antibacterial activity. A 1 ml sample of each was mixed 1:1 with DS14G diluent for testing anti-sperm activity. The preparation from void volume peak was designated HMW_{wd} and the large peak designated LMW_{wd}.

Testing for antibacterial activity

The antibacterial activity of the peaks in Fig. 10 was determined. Activity was found in the HMW_{wd} but not in the LMW_{wd} (results are shown in Table 5A).

Test for anti-sperm activity

A preliminary study indicated that the whole diffusate had an adverse effect on motility of sperm (Table 2). It was thought that the separation of the component according to their molecular weights may separate the beneficial factor(s) from the detrimental factor(s).

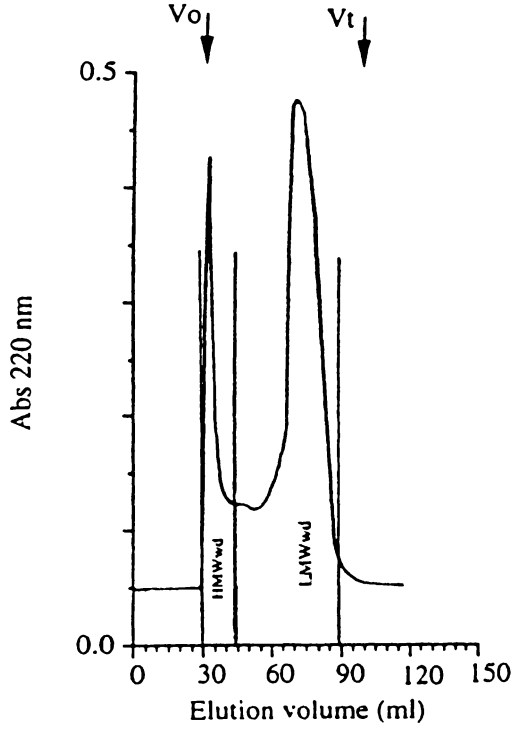


Fig. 10: Gel permeation chromatography of the water diffusate
Gel: Sephadex G25 SF
Sample: 4.5 ml of the concentrated material adjusted to pH 1.7
Eluent: 0.02 mol/l HCl, pH 1.7.
Flow rate: 1 ml/min
Fraction volume: 5 ml

From the prepared samples of HMW_{wd} and LMW_{wd} mentioned above, a 100 µl sample of each was added to 900 µl of a suspension of washed sperm in 14G diluent containing 0.1% bovine serum albumin to give a final concentration of the fraction of 10% and a sperm concentration of $10\text{-}12x10^6$ sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are given in Table 5B.

The HMW_{wd} fraction had an adverse effect on the motility of the sperm. The reduction in motile sperm was significantly greater than that resulting from whole diffusate of seminal plasma against water and whole seminal plasma. Washed sperm that were incubated with the HMW_{wd} showed agglutination in the area between the head and the middle piece. This effect was not observed when sperm were incubated with whole diffusate of seminal plasma dialysed against water. No anti-sperm activity was seen in the LMW_{wd} fraction.

Table 5. Antibacterial and anti-sperm effects of the diffusate, and its fractions, from seminal plasma dialysed against water
A: effect on *E. coli*
Values are mean ± s.e.m (n=3).

Sample	Mean Clearance Zone (mm^2)
Whole diffusate*	23.75 ± 4.2^c
HMW_{wd}	32.3 ± 3.7^b
LMW_{wd}	not detectable
Whole seminal plasma*	69.7 ± 5.7^a

B: Effect on motility of washed bull sperm.
The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		76.7 ± 3.3^a	68.3 ± 1.7^a
HMW_{wd}	0.16	68.4 ± 4.4^a	43.3 ± 6.7^c
LMW_{wd}	0.01	76.7 ± 3.3^a	70.0 ± 2.9^a
Whole diffusate*	0.20	76.7 ± 3.3^a	61.7 ± 3.3^b
Whole seminal plasma*	4.2	76.7 ± 3.3^a	60.0 ± 2.9^b

**At 10% concentration for comparison.*
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

3.2.3.4 SUMMARY AND CONCLUSION:

The diffusate contains components of relatively low molecular weight as shown by gel permeation chromatography and SDS-PAGE under reducing conditions. These components possess antibacterial as well as anti-sperm activity. The diffusate could possibly contain seminalplasmin which has known antibacterial and anti-sperm activity (see Chapter One).

The overall anti-sperm activity increased after the fractionation on Sephadex G25 under acidic conditions. The HMW_{wd} fraction exhibited high anti-sperm activity. The LMW_{wd} did not show detrimental effects on the motility of sperm. It cannot be said that this fraction does not possess any detrimental components, as any effect may be masked by the domination of the beneficial component(s) reported by Baas *et al.* (1983). This beneficial factor did not inhibit the adverse effect of HMW_{wd} since the whole diffusate showed an adverse effect on sperm motility. However, the adverse effect of the whole diffusate was less than that of the HMW_{wd} fraction, probably due to the presence of the beneficial component(s). Nevertheless, the agglutination effect was not seen in the whole diffusate.

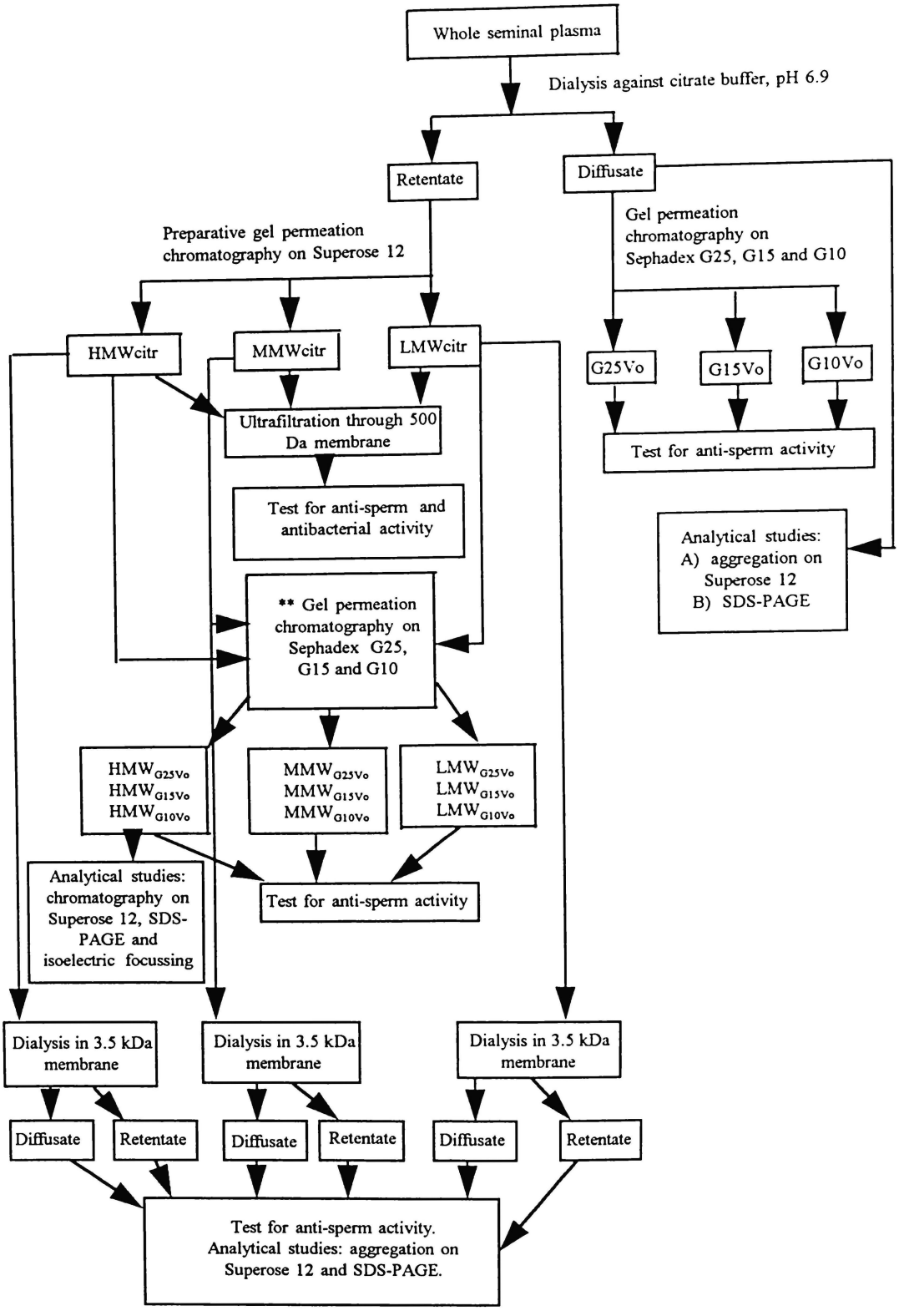
3.3 FURTHER ATTEMPTS TO INVESTIGATE THE LOW MOLECULAR WEIGHT COMPONENTS IN BOVINE SEMINAL PLASMA

3.3.1 DIALYSIS OF SEMINAL PLASMA AGAINST CITRATE BUFFER

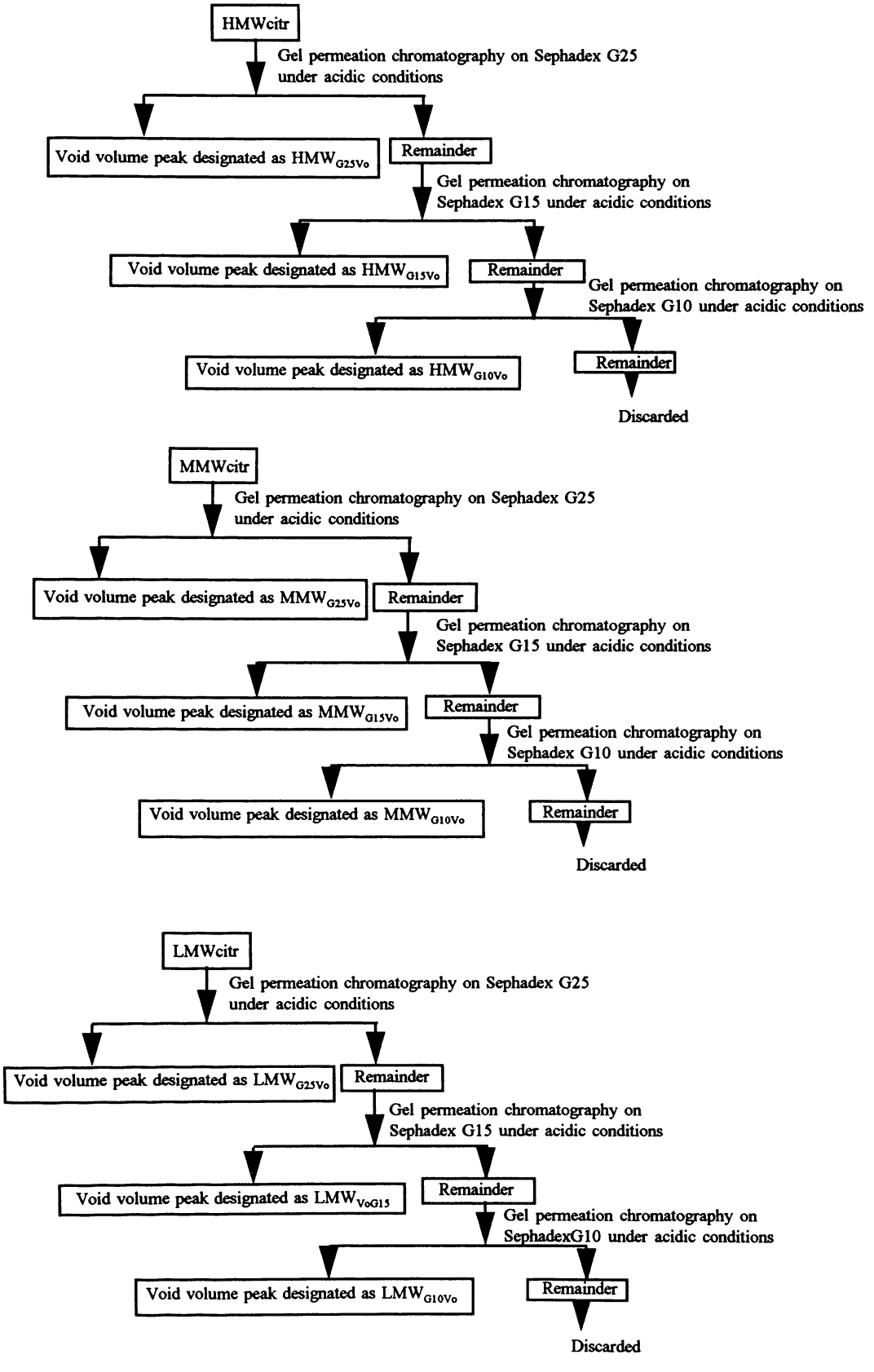
The work in the previous section demonstrated that low molecular weight fractions are the most detrimental to sperm motility. Dialysis against water under neutral conditions did not completely cause the dissociation of the anti-sperm activity from the macromolecule and a substantial amount remains in an aggregated form.

Shannon and Curson (1983) and Garcia and Graham (1987) have reported that dialysis of semen (with a 12 kDa cut-off membrane) in the presence of citrate was beneficial to sperm survival. The beneficial effect of dialysis was attributed to the removal of low molecular weight components from seminal plasma which were assumed to have detrimental effects on sperm, however, the nature of these low molecular weight components was not determined. Shannon *et al.* (1987) reported that dialysis with citrate caused major dissociation of the antibacterial activity from the high molecular weight aggregates. Thus, the nature and origin of these low molecular weight components that appear in the diffusate were further investigated.

Flow chart for Section 3.3



** See next page


HMWcitr
Gel permeation chromatography on Sephadex G25 under acidic conditions
Void volume peak designated as HMW_{G25Vo}
Remainder
Gel permeation chromatography on Sephadex G15 under acidic conditions
Void volume peak designated as HMW_{G15Vo}
Remainder
Gel permeation chromatography on Sephadex G10 under acidic conditions
Void volume peak designated as HMW_{G10Vo}
Remainder
Discarded
MMWcitr
Gel permeation chromatography on Sephadex G25 under acidic conditions
Void volume peak designated as MMW_{G25Vo}
Remainder
Gel permeation chromatography on Sephadex G15 under acidic conditions
Void volume peak designated as MMW_{G15Vo}
Remainder
Gel permeation chromatography on Sephadex G10 under acidic conditions
Void volume peak designated as MMW_{G10Vo}
Remainder
Discarded
LMWcitr
Gel permeation chromatography on Sephadex G25 under acidic conditions
Void volume peak designated as LMW_{G25Vo}
Remainder
Gel permeation chromatography on Sephadex G15 under acidic conditions
Void volume peak designated as LMW_{VoG15}
Remainder
Gel permeation chromatography on SephadexG10 under acidic conditions
Void volume peak designated as LMW_{G10Vo}
Remainder
Discarded

Citrate (a poly-anionic agent) is believed to cause disaggregation of the protein aggregates in bovine seminal plasma through the strong ionic interaction with the cationic components (Shannon *et al.*, 1987). The premise of the following study was, dialysis in the presence of citrate may result in the dissociation of the anti-sperm activity from the macromolecules.

Accordingly, sodium citrate was added to 10 ml of fresh seminal plasma to a concentration of 0.1 mol/l and the pH was adjusted to 6.9. The seminal plasma was then placed in a dialysis bag (19 mm flat width) with a molecular weight cut-off of 12 kDa and dialysed against 12 volumes of 0.1 mol/l sodium citrate buffer at pH 6.9 for 18 h at 5°C with continuous stirring.

3.3.2 A STUDY OF THE DIFFUSATE OF SEMINAL PLASMA DIALYSED AGAINST CITRATE BUFFER

3.3.2.1 GEL PERMEATION CHROMATOGRAPHY OF THE DIFFUSATE OF SEMINAL PLASMA ON SEPHADEX COLUMNS

After 18 h of dialysis, the retentate in the dialysis bag was removed and retained for further investigation. The diffusate was collected and the volume reduced by rotary evaporation to the original volume of the seminal plasma (10 ml). A 1 ml sample of the concentrate was retained for analytical studies and the pH of the rest of the concentrated diffusate was adjusted to 1.7 by the addition of 1 mol/l HCl, then 4.5 ml was chromatographed on a Sephadex G25SF column eluted with 0.02 mol/l HCl, pH 1.7 as detailed in Fig. 11. The chromatography was repeated again. A peak at the void volume, with an estimated molecular weight of 5 kDa or greater, was followed by a large peak. The fractions comprising the void volume peak from both runs were pooled, the pH adjusted to 7, and rotary evaporated to 4.5 ml (half the original volume). This peak was designated G25Vo.

The fractions comprising the large peak from 45 to 100 ml in Fig. 11 from both runs were collected and rotary evaporated to 9 ml. The concentrate was centrifuged at 20 000 *g* for 10 min and a 4.5 ml sample was chromatographed on a column of Sephadex G15, eluted with 0.02 mol/l HCl as detailed in Fig. 12. The results (shown in Fig. 12) indicated that the large peak from Fig. 11 contained components eluting at the void volume with estimated molecular weights of 1.5 kDa and greater, followed by a large peak near the bed volume. The chromatography was repeated once more. The fractions comprising the void volume peak from both runs were collected, the pH

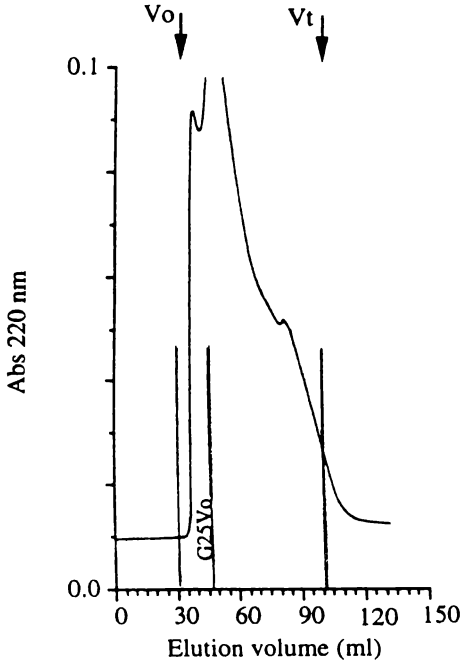


Fig. 11: Gel permeation chromatography of the diffusate of seminal plasma dialysed against citrate buffer.
Gel: Sephadex G25 SF
Sample: 4.5 ml of the concentrated material adjusted to pH 1.7
Eluent: 0.02 mol/l HCl, pH 1.7
Flow rate: 1 ml/min
Fraction volume: 5 ml

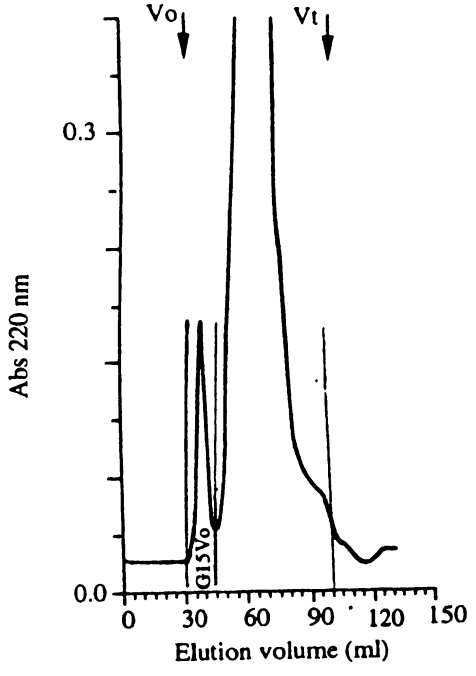


Fig. 12

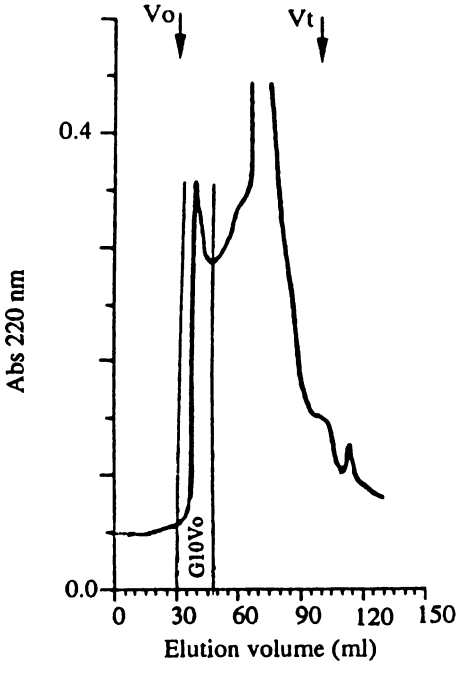


Fig. 13

Fig. 12: Gel permeation chromatography of the material in the second peak in Fig. 11.
Gel: Sephadex G15
Sample: 4.5 ml of the concentrated material adjusted to pH 1.7
Eluent: 0.02 mol/l HCl, pH 1.7
Flow rate: 1 ml/min
Fraction volume: 5 ml

Fig. 13: Gel permeation chromatography of the material in the second peak in Fig. 12.
Gel: Sephadex G10
Sample: 4.5 ml of the concentrated material adjusted to pH 1.7
Eluent: 0.02 mol/l HCl, pH 1.7
Flow rate: 1 ml/min
Fraction volume: 5 ml.

adjusted to 7, and reduced to 4.5 ml (half the volume of the original sample) by rotary evaporation and designated G15Vo.

The second peak from 45 to 100 ml in Fig. 12 was processed on Sephadex G10 as above (Fig. 13) and the fractions comprising the void volume peak were reduced to 4.5 ml (half the volume of the original sample) by rotary evaporation and designated G10Vo.

Test for anti-sperm activity in the void volume peaks from the chromatography of the diffusate on Sephadex columns

All the void volume peaks from the Sephadex columns shown in Figs. 11, 12, and 13 were desalted on a Sephadex G10 column eluted with water. The desalted fractions were concentrated to 4.5 ml (half the original volume), and the protein concentration determined. The fractions were then mixed with an equal volume of DS14G and tested for anti-sperm activity.

From the prepared samples mentioned above, a 100 µl sample of each was added to 900 µl of a suspension of washed sperm in 14G diluent containing 0.1% bovine serum albumin to give a final concentration of the fraction of 10% and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are given in Table 6.

Table 6. The effect of the void volume peaks in Figs. 11, 12 and 13 on the motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately and after 18 h incubation at ambient temperature. Values are mean ± s.e.m (n=4). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control		82.5±1.4^a	71.3±2.4^a
G25Vo	0.336	71.5±2.4^b	46.3±2.4^c
G15Vo	0.06	81.3±1.3^a	68.8±1.3^a
G10Vo	0.22	65.0±2.0^c	37.5±1.4^d
*DSP	3.63	81.3±1.3^a	61.3±2.4^b

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison.*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The results, shown in Table 6, indicated marked anti-sperm activity in G25Vo and G10Vo while G15Vo did not differ from the control of 14G diluent alone. Addition of the G25Vo and G10Vo fractions immediately reduced the percentage of motile sperm compared with the control (see Table 6). The relative amount of protein in each fraction tested differed, and in the case of G15Vo was significantly less than in the other two.

Concentration-dependent effects of the void volume peaks from the chromatography of the diffusate on Sephadex columns

To test a dose response effect of the individual void volume fractions, aliquots of 100, 200 or 400 µl of the fraction under test were made up to 1 ml final volume of reaction mixture to give 10, 20 and 40% concentration of the fraction and 10-12 x 10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 7.

Table 7. Effect of various concentrations of the void volume fraction in Figs. 11, 12 and 13 on the motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=4). The concentrations shown are the percentage of the original concentration of the fractions in seminal plasma.

Treatment	Concentration (v/v)%	Protein Conc. (mg/ml)	% Motile	
			0 h	18 h
Control (diluent)			81.2 ±2.5^a	65.0±2.8^a
G25 Vo	10	0.336	72.5±2.9^b	46.6±5.8^{bc}
	20	0.672	65.0±4.1^c	9.2±1.5^d
	40	1.34	46.0±7.5^d	2.0±2.4^e
G15 Vo	10	0.06	81.2±2.5^a	67.5±3.5^a
	20	0.12	81.2±2.8^a	47.5±3.6^b
	40	0.24	77.5±2.9^a	45.0±4.1^{bc}
G10 Vo	10	0.22	65.0±5.8^c	38.7±2.5^c
	20	0.44	52.5±3.5^d	4.5±1.0^e
	40	0.88	47.5±3.5^d	3.5±1.0^e

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

It is evident from the results in Table 7 that the detrimental effect was enhanced with higher concentrations of the respective fractions.

3.3.2.2 GEL PERMEATION CHROMATOGRAPHY OF THE CITRATE DIFFUSATE TO INVESTIGATE RE-AGGREGATION

To determine whether the components in the citrate diffusate have the capacity to form aggregates higher than 12 kDa (*i.e.* the cut-off of the dialysis membrane) the composition of the citrate diffusate was further investigated. A 500 µl sample of the concentrated citrate diffusate was mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7 and chromatographed on an analytical Superose 12 column eluted with 0.1 mol/l of Tris-HCl at pH 7, as detailed in Fig. 14. Even though the components in the diffusate had passed though a membrane with a molecular weight cut-off of 12 kDa, peaks were detectable in regions higher than 12 kDa up to an estimated molecular weight of 80 kDa (Fig. 14). This indicated that molecular aggregation had occurred.

3.3.2.3 SDS-PAGE OF THE DIFFUSATE OF SEMINAL PLASMA DIALYSED AGAINST CITRATE BUFFER

The composition of the diffusate of seminal plasma dialysed against citrate buffer was further investigated by SDS-PAGE under reducing conditions performed on a 20% polyacrylamide gel. A 10 µl sample of the concentrated diffusate of seminal plasma dialysed against citrate buffer was mixed 1:1 with 5 times concentrated sample buffer and loaded on the gel. The results in Fig. 15 show that the diffusate of seminal plasma dialysed against citrate buffer contained proteins with estimated molecular weights ranging between 2 and 16 kDa. There was a major band observed in the region of 5 kDa and a faint band of molecular weight lower than 2 kDa.

3.3.2.4 SUMMARY AND CONCLUSION:

Dialysis of seminal plasma against citrate buffer resulted in the appearance of low molecular weight components in the diffusate. Compared with the dialysis against water (Table 5b *cf* Table 6) it can be seen that the diffusate resulting from the dialysis against citrate contains more proteins or peptides. The diffusate of seminal plasma dialysed against citrate buffer contained components shown to be detrimental to sperm motility. Compared with the control and with the retentate of seminal plasma dialysed against water, the effect was significant ($p<0.05$). This detrimental effect on sperm motility was concentration-dependent. The presence of low molecular weight components was evident from gel permeation chromatography and SDS-PAGE of the

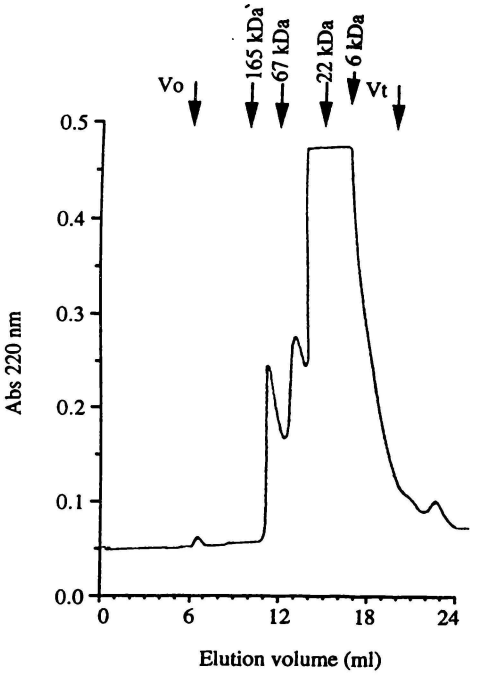


Fig. 14

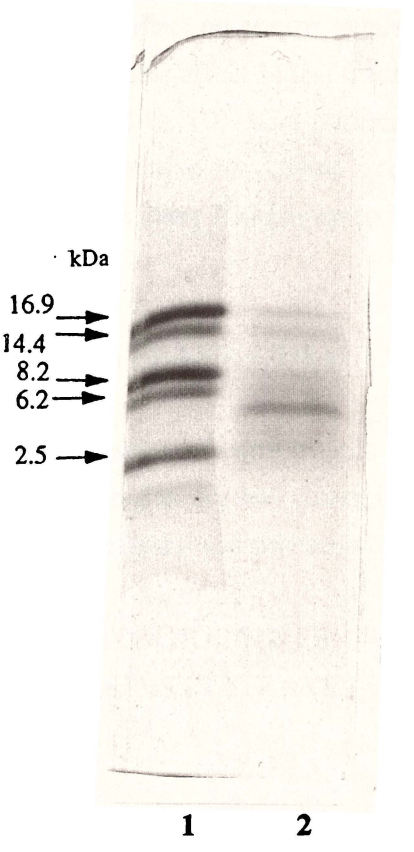


Fig.15

Fig. 14: Gel permeation chromatography of the citrate diffusate.
Column: analytical Superose 12 HR10/30.
Sample: 500 µl of the concentrated diffusate mixed 1:1 with 0.2 mol/l Tris-HCl, at pH 7.0
Eluent: 0.1 mol/l Tris- HCl buffer, pH 7.0.
Flow rate: 0.5 ml/min

Fig. 15: SDS-PAGE under reducing conditions of the citrate diffusate on a 20% polyacrylamide gel.
Lane 1: molecular weight markers. Lane 2: citrate diffusate.

diffusate. Several bands of molecular weight less than 10 kDa were seen on the gel, more prominently at the region of estimated molecular weight of 5-8 kDa.

Citrate as a disaggregating agent caused the release of low molecular weight components from the macromolecular components. These results are in agreement with the observations of Shannon *et al.* (1987). The effect of citrate was evident from the increase in protein concentration in the diffusate compared with the level of protein observed following dialysis against water. Citrate caused the appearance of components of molecular weight lower than the exclusion size of the Sephadex G15 column, *i.e.* less than 1.5 kDa, which exerted a detrimental effect on sperm, while dialysis against water in the absence of citrate did not show this effect in the fractions that eluted in the peak near the bed volume (*i.e.* LMW_{wd} in Fig. 10). Gel permeation chromatography of the citrate diffusate on the analytical Superose 12 column under neutral conditions showed aggregation into the region higher than 12 kDa up to 80 kDa.

3.3.3 A STUDY OF THE RETENTATE OF SEMINAL PLASMA DIALYSED AGAINST CITRATE BUFFER

Dialysis of seminal plasma against citrate buffer caused diffusion of components with anti-sperm activity. To determine whether all the anti-sperm activity had dissociated from the macromolecule, the retentate resulting from this dialysis was investigated.

3.3.3.1 PREPARATIVE GEL PERMEATION CHROMATOGRAPHY OF THE RETENTATE OF SEMINAL PLASMA DIALYSED AGAINST CITRATE BUFFER

The retentate of seminal plasma dialysed against citrate buffer (Section 3.3.1) was centrifuged at 20 000 *g* and a 1 ml sample was injected onto the preparative Superose 12 column and eluted with 0.1 mol/l citrate buffer, at pH 6.9. Citrate was used as a disaggregating agent. Repeated chromatography (run 10 times) was conducted to accumulate sufficient material for further studies. The results from each run were identical and shown in Fig. 16. Three major peaks covering a wide range of molecular weights were obtained. However, the bulk of the material was eluted between 45 and 75 ml corresponding to molecular weights between 20 and 200 kDa. The material that eluted from the column was divided into three molecular weight ranges:

- from 30 to 45 ml, designated HMWcitr
- from 45 to 75 ml, designated MMWcitr

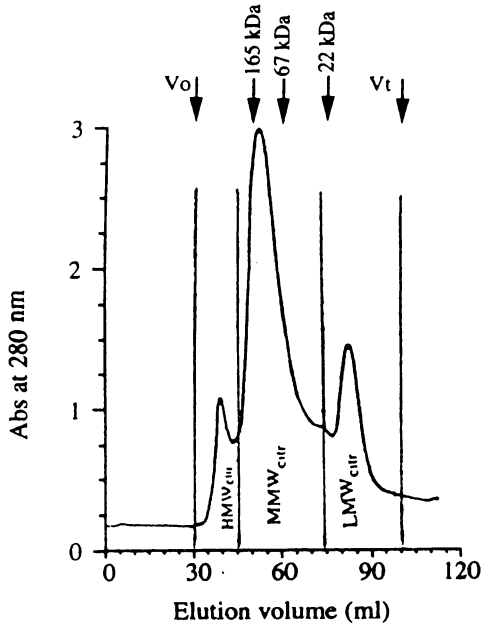


Fig. 16: Gel permeation chromatography of the retentate of seminal plasma dialysed against citrate buffer
Column: Preparative Superose 12.
Sample: 1 ml of the retentate in 0.1 mol/l citrate buffer at pH 6.9.
Eluent: 0.1 mol/l citrate buffer, pH 6.9
Flow rate: 1 ml/min
Fraction volume: 5 ml

- from 75 to 100 ml, designated LMWcitr

The fractions that comprised each area were pooled, and concentrated by rotary evaporation to 10 ml (the original volume of seminal plasma). The protein concentration of each area shown in Fig. 16, after it had been concentrated, and prior to ultrafiltration or dialysis, was:

- HMWcitr: 4.3 mg/ml
- MMWcitr: 21.6 mg/ml
- LMWcitr: 7.2 mg/ml

These concentrates were each divided into two parts and stored frozen for further processing.

Preparation of samples for antibacterial and anti-sperm activity testing

Ultrafiltration of the HMWcitr, MMWcitr and LMWcitr

One half (5 ml) of the concentrates of HMWcitr, MMWcitr and LMWcitr were desalted by ultrafiltration through a membrane with molecular weight cut-off of 500 Da with the addition of 500 ml of Milli Q water. The retentates (5 ml) were concentrated by rotary evaporation to 2.5 ml. A 1 ml sample was retained for further studies, 0.5 ml was diluted 1:1 with 0.2 mol/l citrate buffer to test for antibacterial activity and the rest was then diluted 1:1 with DS14G diluent to test for anti-sperm activity.

Test for antibacterial activity

Samples of the ultrafiltered fractions of HMWcitr, MMWcitr and LMWcitr were tested for antibacterial activity. Antibacterial activity was found in all these fractions. For the HMWcitr and MMWcitr, the antibacterial activity (61.4±4.5 mm^2) was equal to the standard (dialysed bovine seminal plasma), while the LMWcitr had less antibacterial activity (34±3.3 mm^2).

There was a marked increase in antibacterial activity as a result of dialysis and chromatography since these fractions were tested at their original concentration in seminal plasma.

Test for anti-sperm activity

From the prepared samples of the ultrafiltered fractions of HMWcitr, MMWcitr and LMWcitr, a 100 µl aliquot was added to 900 µl of washed sperm suspended in 14G

diluent to give a 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 8.

Table 8. Effect of the ultrafiltered fractions HMWcitr, MMWcitr and LMWcitr from Fig. 16 on the motility of washed bull sperm.
The values for motility shown are the percentage of motile sperm immediately after addition and after 18 hours incubation at 18 to 21°C. Values are mean ± s.e.m. (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		81.2 ±2.9[a]	75.0±2.9[a]
HMWcitr	0.43	81.7±3.5[a]	70.0±5.0[a]
MMWcitr	2.16	80.7±2.3[a]	71.7±2.9[a]
LMWcitr	0.72	78.3±2.8[a]	43.3±2.8[c]
DSP*	3.63	78.3±2.9[a]	61.7±1.7[b]

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison.*
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The results shown in Table 8 indicate that the LMWcitr reduced the percentage of motile sperm after 18 h incubation, while the HMWcitr and MMWcitr were not different from the control. However, the anti-sperm activity was significantly lower in the HMWcitr fraction than in the LMWcitr fraction. Although there was no significant inhibition of sperm motility soon after addition of the LMWcitr fraction, inhibition was quite marked after an 18 h incubation at ambient temperature. The anti-sperm activity in the LMWcitr fraction was greater than that in seminal plasma dialysed against water (DSP).

Dialysis of HMWcitr, MMWcitr and LMWcitr

The second part of the fractions HMWcitr, MMWcitr and LMWcitr in Fig. 16 that had been concentrated by rotary evaporation (5 ml) was dialysed using dialysis tubing with a molecular weight cut-off of 3.5 kDa. The dialysis was conducted to remove citrate and to see if the low molecular weight component would diffuse out due to further disaggregation. These fractions were dialysed against 2 L of Milli Q water for 24 hours with continuous stirring. Each of the retentates was then concentrated down to 2.5 ml by rotary evaporation. A 1 ml sample of each concentrate was diluted 1:1 with DS14G diluent for testing of the anti-sperm activity and 0.5 ml was mixed

1:1 with 0.2 mol/l citrate buffer at pH 6.9 for testing the antibacterial activity. The diffusates were retained for further experiments.

Activity of the retentate of dialysed HMWcitr, MMWcitr and LMWcitr

Test for antibacterial activity

A sample of the dialysed fractions HMWcitr, MMWcitr and LMWcitr was tested for antibacterial activity. The results showed a mean clear zone of 53.8±4.0 mm^2 for HMWcitr and MMWcitr and a mean clear zone of 61.4±4.5 mm^2 for the standard (dialysed bovine seminal plasma), while LMWcitr had relatively less antibacterial activity (32±4.0 mm^2). These results indicated that antibacterial activity was present in all of these fractions, however, the activity was less than that observed in the ultrafiltered fractions. It appears that dialysis may have caused some loss in antibacterial activity.

Test for anti-sperm activity

From the prepared samples of the dialysed fractions HMWcitr, MMWcitr and LMWcitr, 100 µl was added to 900 µl of washed sperm suspended in 14G diluent to give 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 9.

Table 9. Effect of the retentate of dialysed HMWcitr, MMWcitr and LMWcitr fractions of seminal plasma from Fig. 16 on the motility of washed bull sperm. *The values for motility are the percentage of motile sperm immediately, and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.*

Treatment	Protein conc. (mg/ml)	% Motile	
		0 hours	18 hours
Control (diluent)		81.6±1.7^a	71.7±1.7^a
Retentate of dialysed HMWcitr	0.30	81.6±1.7^a	63.3±1.7^b
Retentate of dialysed MMWcitr	1.26	81.6±1.7^a	65.0±2.9b
Retentate of dialysed LMWcitr	0.48	76.6±1.8^b	41.8±1.9^c
DSP*	3.63	81.6±1.7^a	63.4±1.5^b

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison.*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

Compared with the results after ultrafiltration (Table 8), it is clear that dialysis of these fractions did not reduce the anti-sperm activity. All fractions reduced the

percentage of motile sperm after 18 h incubation, markedly so with the fraction of low molecular weight. However, activity was greater in the dialysed MMWcitr compared with the ultrafiltered MMWcitr.

A study of the diffusate obtained from the dialysis of HMWcitr, MMWcitr and LMWcitr

The diffusates obtained from the dialysis of HMWcitr, MMWcitr and LMWcitr were each concentrated by rotary evaporation to 5 ml (the original volume of the samples) and desalted on a column of Sephadex G10 eluted with water to remove citrate from the fractions. The fractions comprising the void volume peak from this chromatography were collected, pooled and concentrated to half the original volume (2.5 ml) by rotary evaporation. A 1 ml sample of each was then diluted 1:1 in DS14G for testing the anti-sperm activity.

Test for anti-sperm activity

A 100 µl sample of each of the prepared diffusates of HMWcitr, MMWcitr and LMWcitr was added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 10.

Table 10. Effect of the diffusates from the HMWcitr, MMWcitr and LMWcitr fractions of seminal plasma (desalted on Sephadex G10) on the motility of washed bull sperm.

The values for motility are percentage of motile sperm immediately, and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		81.6±1.7^a	70.0±2.9^a
Diffusate of HMWcitr	0.11	71.8±3.3^b	48.3±4.4^c
Diffusate of MMWcitr	0.83	68.3±4.4^{bc}	40.0±2.9^d
Diffusate of LMWcitr	0.20	65.0±2.9^c	36.7±1.7^d
DSP*	3.63	80.0±0.0^a	63.4±1.7^b

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison.*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The results in Table 10 indicate that the material that diffused out of the dialysis membrane had an adverse effect on sperm motility. The reduction in the percentage

of motile sperm was significant in all diffusates. Interestingly, the material that diffused out from the various fractions possessed higher anti-sperm activity than the original fractions themselves, noticeably so in the case of MMWcitr (see Table 8). The separation of the low molecular weight components from the high molecular weight components seems to enhance the anti-sperm activity.

SDS-PAGE of the diffusates from dialysis of HMWcitr, MMWcitr and LMWcitr

The diffusates from dialysis of the fractions from Fig. 16 that had passed through dialysis membrane of 3.5 kDa molecular weight cut-off appeared to have high anti-sperm activity. To gain a better idea about their composition and the molecular weight of the components, they were subjected to SDS-PAGE under reducing conditions. Preparation of samples and electrophoresis was performed as described in Chapter Two. A 10 µl sample of each was loaded onto a 20% polyacrylamide gel. The results in Fig. 17 show that the diffusate of the dialysed fractions from Fig. 16 contained components of low molecular weight as well as some components of molecular weight much higher than the cut-off of the membrane (3.5 kDa). It appeared that components of the diffusates had aggregated to form larger molecules. A similar observation was reported by Shannon *et al.* (1987). They presented evidence that showed that the antibacterial activity in seminal plasma which passed through a dialysis membrane with a 3.5 kDa molecular weight cut-off in the presence of citrate or under acidic conditions reaggregated to higher molecular weight components.

Gel permeation chromatography under neutral conditions of the diffusates from dialysis of HMWcitr, MMWcitr and LMWcitr

To determine the behaviour of the material that passed through a membrane with a molecular weight cut-off of 3.5 kDa under neutral conditions, it was subjected to chromatography on a Superose 12 column. Accordingly, 250 µl of the concentrated diffusate of each of HMWcitr, MMWcitr and LMWcitr was mixed 1:1 with 0.2 mol/l Tris-HCl buffer at pH 7 and incubated for 2 h at 37°C then chromatographed on the Superose 12 column eluted with 0.1 mol/l Tris-HCl buffer at pH 7. The results are shown in Fig. 18. The elution profile of the three chromatograms indicated the presence of components with molecular weights greater than 6 kDa up to 60 kDa. The component(s) in the diffusates is thus likely to be a molecule or a complex of molecules which has the ability to reaggregate to higher molecular weight.

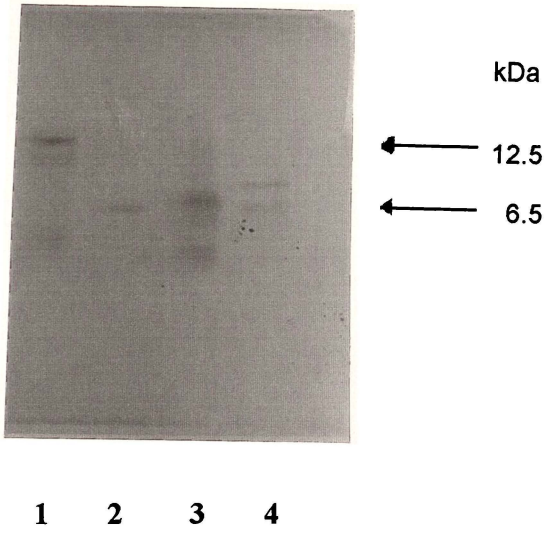


Fig. 17: SDS-PAGE under reducing conditions of the material appearing in the diffusate resulting from the dialysis of HMWcitr, MMWcitr and LMWcitr in dialysis membrane with 3.5 kDa molecular weight cut-off.
Lane 1: diffusate of MMWcitr. Lane 2: aprotinin (6.5 kDa). Lane 3: diffusate of LMWcitr. Lane 4: diffusate of HMWcitr.

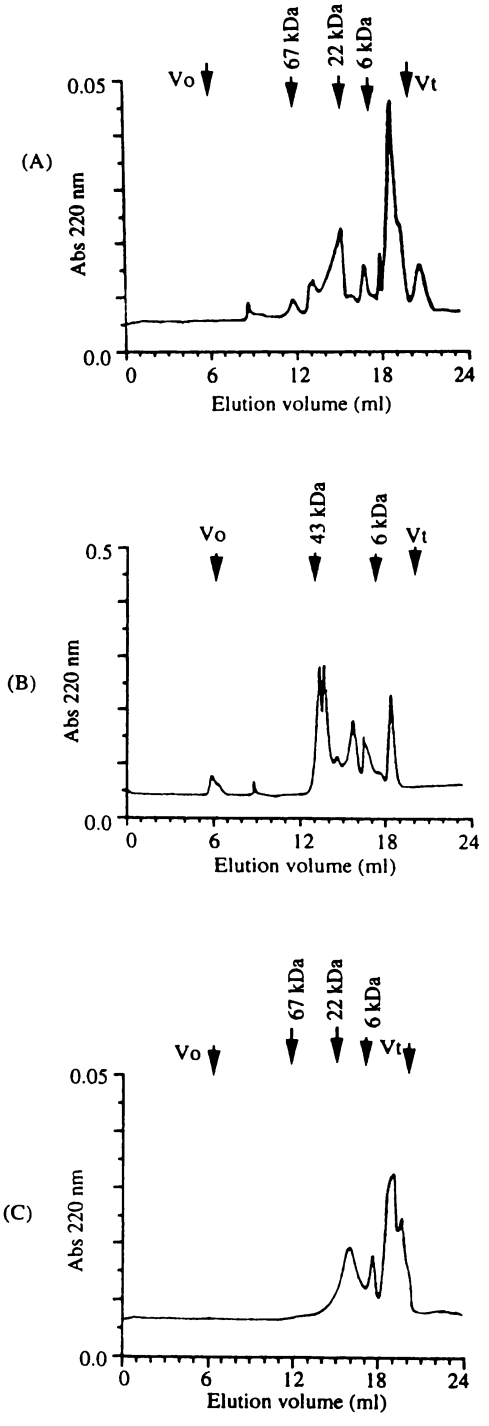


Fig. 18: Gel permeation chromatography of the diffusates that resulted from the dialysis in a membrane with 1 kDa molecular weight cut-off,
a) diffusate of HMWcitr,
b) diffusate of MMWcitr,
c) diffusate of LMWcitr
Column: analytical Superose 12 HR10/30.
Sample: 250 µl of a, b, or c mixed 1:1 with 0.2 mol/l Tris -HCl, pH 7.0
Eluent: 0.1 mol/l Tris- HCl buffer, pH 7.0
Flow rate: 0.5 ml/min

Further study of HMWcitr, MMWcitr and LMWcitr

Acid dissociation of the HMWcitr

Dialysis of HMWcitr in the presence of the contained citrate showed that low molecular weight components are released and appear in the diffusate. This may be due to the aggregates of high molecular weight being in equilibrium with the low molecular weight component(s); removal of the low molecular weight component(s) therefore creating a new equilibrium. To determine whether chromatography of this high molecular weight component under acid conditions would produce low molecular weight components similar to those observed from the dialysis experiment, gel permeation chromatography was performed on a Sephadex column. Accordingly, more of the fraction was generated by dialysis of 5 ml of seminal plasma as in Section 3.3.1 followed by chromatography as shown in Fig. 16 (Section 3.3.3.1). The pH of the fractions comprising the HMWcitr (5 ml) in Fig. 16 was adjusted to pH 1.7 with HCl and subjected to chromatography on Sephadex G25, G15 and G10 as described for the citrate diffusate (Section 3.3.1.1). Low pH was chosen to suppress the charges on the molecule and cause further release of low molecular weight components. The fractions that comprised the void volume peaks were collected, and the pH adjusted to 7. They were then rotary evaporated to 2.5 ml (half the original volume of seminal plasma). These fractions were designated HMW_{G25Vo}, HMW_{G15Vo} and HMW_{G10Vo} respectively.

Acid dissociation of the MMWcitr

The fractions of the MMWcitr were processed in the same manner as with the HMWcitr. The void volume peaks from chromatography on these columns were designated as MMW_{G25Vo} for the void volume peak from Sephadex G25, MMW_{G15Vo} for the void volume peak from Sephadex G15 and MMW_{G10Vo} for the void volume peak from Sephadex G10.

Acid dissociation of the LMWcitr

A similar procedure was followed in processing the LMWcitr as with HMWcitr and the MMWcitr. The void volume peaks from chromatography on the columns were designated as: LMW_{G25Vo} for the void volume peak from Sephadex G25, LMW_{G15Vo} for the void volume peak from Sephadex G15 and LMW_{G10Vo} for the void volume peak from Sephadex G10.

All void volume peaks were reduced to 2.5 ml by rotary evaporation and then 1 ml of each was diluted 1:1 with DS14G diluent for testing anti-sperm activity.

Test for anti-sperm activity of the void volume peaks obtained from the acid dissociation of HMWcitr, MMWcitr and LMWcitr

From the prepared samples mentioned above, aliquots were added to the reaction mixture to achieve 10 and 20% concentration in a final reaction mixture of 1 ml containing 10-12x10^6 sperm/ml. The reaction mixture was incubated for 18 hours at ambient temperature. Visual assessment of motility was done immediately after the additions and after 18 hour incubation. The results are shown in Tables 11, 12 and 13.

Table 11. Effect of the void volume peaks from gel permeation chromatography on Sephadex G25, G15 and G10 of the HMWcitr fractions on the motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately and after 18 h incubation at ambient temperature. Values are mean ± s.e.m (n=3). The concentrations shown are the percentage of the original concentration of the fractions in seminal plasma.

Treatment	Conc. (v/v)%	Protein conc. (mg/ml)	% Motile	
			0 hrs	18 hrs
Control (diluent)			80.0 ±0.5^a	76.1±2.8^a
DSP*	10	3.63	78.3±2.9^a	73.3±2.9^a
	20	7.2	78.3±2.9^a	41.7±2.9^d
HMW_{G25Vo}	10	0.21	78.4±3.1^a	71.7±3.0^b
	20	0.42	71.8±3.3^b	51.7±2.9^c
HMW_{G15Vo}	10	0.06	68.3±1.6^b	56.7±5.8^c
	20	0.12	61.7±1.7^d	25.0±5.0^e
HMW_{G10Vo}	10	0.11	70.0±5.0^b	55.0±5.0^c
	20	0.22	65.0±0.0^c	33.3±4.9^d

**The retentate of dialysed seminal plasma was added for comparison*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

Table 12. Effect of the void volume peaks from gel permeation chromatography on Sephadex G25, G15 and G10 of the MMWcitr fraction on the motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately and after 18 h incubation at ambient temperature. Values are mean ± s.e.m (n=3). The concentrations shown are the percentage of the original concentration of the fractions in seminal plasma.

Treatment	Conc. (v/v)%	Protein conc. (mg/ml)	% Motile	
			0 hrs	18 hrs
Control			80.0±0.0^{a}	76.7±3^{a}
DSP*	10	3.63	78.3±2.9.^{a}	68.3±2.9^{bc}
	20	7.2	78.3±3.2^{a}	41.7±4.0^{e}
MMW_{G25Vo}	10	1.57	78.4±54^{a}	71.7±4.0^{ab}
	20	3.14	78.3±03.^{a}	51.7±2.9^{d}
MMW_{G15Vo}	10	0.21	56.7±13.^{b}	36.7±5.8^{e}
	20	0.42	51.5±5.8^{b}	21.8±5.0^{f}
MMW_{G10Vo}	10	0.23	80.0±0.0^{a}	76.6±2.9^{a}
	20	0.46	80.0±0.0^{a}	63.3±2.9^{c}

**The retentate of dialysed seminal plasma for comparison*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

Table 13. Effect of the void volume peaks from gel permeation chromatography on Sephadex G25, G15 and G10 of the LMWcitr fraction of seminal plasma on the motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). The concentrations shown are the percentage of the original concentration of the fractions in seminal plasma.

Treatment	Conc. (v/v) %	Protein Conc. (mg/ml)	% Motile	
			0 h	18 h
Control (diluent)			81.8±1.9^{a}	71.7±1.7^{a}
LMW_{G25Vo}	10	0.34	73.3±1.7^{b}	53.3±4.4^{c}
	20	0.68	66.7±1.9^{c}	38.3±1.6^{e}
LMW_{G15Vo}	10	0.09	70.0±2.9^{bc}	66.7±1.9^{b}
	20	0.18	63.3±1.7^{c}	51.7±1.9^{cd}
LMW_{G10Vo}	10	0.22	58.3±1.8^{d}	41.7±4.5^{e}
	20	0.44	48.4±1.9^{e}	31.7±1.9^{f}
DSP*	10	3.63	80.0±0.0^{a}	65.0±2.9^{b}
	20		80.0±0.0^{a}	48.5±1.8^{d}

**The retentate of dialysed seminal plasma for comparison*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The HMW_{G15V_0} and the HMW_{G10V_0} fractions reduced the percentage of motile sperm soon after addition compared with the control and the unfractionated dialysed seminal plasma and the effect was even greater after 18h incubation at 18°C. In contrast, the HMW_{G25V_0} fraction had no effect on sperm motility soon after addition, but at higher concentrations (20%) and with further incubation, it did markedly reduce sperm motility. The MMW_{G15V_0} (Table 12) showed the same detrimental effects, whereas MMW_{G10V_0} caused no reduction in the percentage of motile sperm soon after addition. All the LMW fractions immediately reduced the percentage of motile sperm after addition and the effect was concentration-dependent. On individual comparisons, after 18 h incubation, LMW_{G25V_0} and LMW_{G10V_0} were significantly more damaging than LMW_{G15V_0}. The unfractionated dialysed seminal plasma, used as a control, also reduced sperm motility after incubation for 18h.

It is interesting that the fractions exhibited markedly greater anti-sperm activity than seminal plasma dialysed against water.

Examination of the composition of the fractions dissociated by citrate and acid from the retentate of dialysis of seminal plasma against citrate

Gel permeation chromatography on Superose 12 under acidic conditions

Chromatography of the retentate of seminal plasma against dialysed citrate buffer is shown in Fig. 16. Fractions obtained from the retentate when chromatographed on the Sephadex columns under acidic conditions, resulted in the appearance of low molecular weight components below 5 kDa (*i.e.* the void volume of Sephadex G15 and G10). It is difficult to determine the nature of the components in the void volume peak of Sephadex G25. It could be an aggregate of 5 kDa or much greater. Therefore the purpose of this experiment was to determine the effect of the low pH on the HMWcitr aggregate (with estimated molecular weight of more than 150 kDa) and the resulting changes in the molecular weight. Accordingly, 100 µl of the concentrate of the HMWcitr was chromatographed on a Superose 12 column eluted with 0.02 mol/l HCl, at pH 1.7. The profile in Fig. 19 shows that the material in this fraction eluted at the region near the bed volume of less than 40 kDa. Clearly, low pH had a substantial effect on the aggregate, causing substantial disaggregation.

Gel permeation chromatography on Superose 12 under neutral conditions

The components that resulted from the chromatography of HMWcitr on a Sephadex column under acidic conditions are disaggregated material (*i.e* HMW_{G15V_0} and

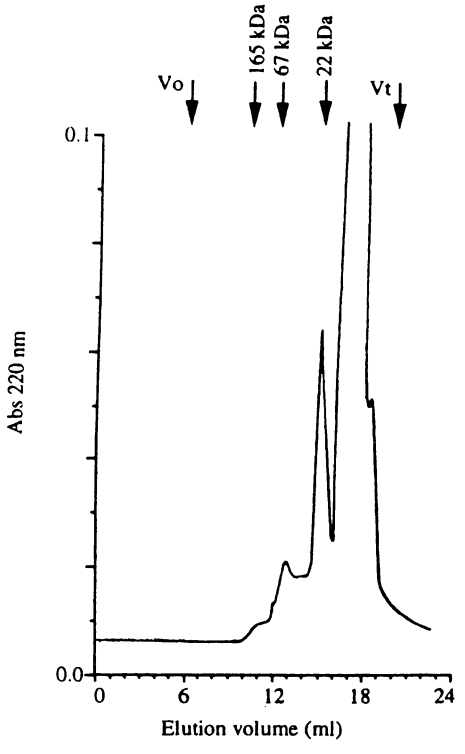


Fig. 19: Gel permeation chromatography of the HMWcitr from Fig. 16.
Column: analytical Superose 12.
Sample: 100 µl of HMWcitr at pH 1.7.
Eluent: 0.02 mol/l HCl, at pH 1.7.
Flow rate: 1 ml/min

HMW_{G10V_0}). Chromatography under neutral conditions was conducted to see if the components in HMW_{G15V_0}, and HMW_{G10V_0} of presumably low molecular weight, which originated from high molecular weight components, remained in a disaggregated form of less than 5 kDa. Accordingly, 250 µl of the concentrates of HMW_{G25V_0}, HMW_{G15V_0} and HMW_{G10V_0} were incubated with 0.2 mol/l Tris-HCl buffer at pH 7 for 2 h at 37°C then chromatographed on the Superose 12 column and eluted with 0.1 mol/l Tris-HCl buffer at pH 7. The results are in Fig 20a, b and c. The elution profiles in the three figures show several peaks of estimated molecular weight higher than the apparent molecular weight on the Sephadex columns, some of them of high molecular weight (up to the void volume of the Superose 12 columns; *i.e.* >150 kDa). It is evident that components in the void volume peaks of the Sephadex G15 and G10 columns have the ability to reaggregate to form components of higher molecular weight than those from which they originated.

SDS-PAGE of the various fractions dissociated by citrate and acid from the retentate of dialysis against citrate buffer

SDS-PAGE under reducing conditions was conducted to investigate the composition and molecular weight of the Sephadex columns void volume peaks obtained from the chromatography of HMWcitr under acidic conditions. Samples were prepared by mixing 10 µl of HMW_{G25V_0}, HMW_{G15V_0}, or HMW_{G10V_0} with equal volume of 5 times concentrated sample buffer. A 1 µl sample of each was loaded onto a 20% high density gel for electrophoresis. The results in Fig. 21 show that all the void volume peaks (*i.e.* HMW_{G25V_0}, HMW_{G15V_0} and HMW_{G10V_0}) were comprised of a complex of components. Several bands with diverse molecular weights were seen. The void volume peak from G25 was found to contain components from a molecular weight of 20 kDa (and probably higher) down to 6 kDa. The void volume peak from G15 also showed that it was still in a complexed state and bands of molecular weight higher than the exclusion size of the column appeared on the gel. The material which was chromatographed on the Sephadex G15 column was that which was eluted after the void volume of Sephadex G25 and was presumed to have a molecular weight of less than 5 kDa, the exclusion size of the Sephadex G25 column. The G10 void volume peak contained components with a diverse molecular weights, with some bands in the region of 2 to 3 kDa. A common band to all fractions was seen in the molecular weight region of 6 kDa. The appearance of bands at the region of estimated molecular weight higher than the exclusion size of the Sephadex G15 and G10 columns (*i.e.* higher than 1.5 kDa) indicated that aggregation had take place even in the presence of SDS.

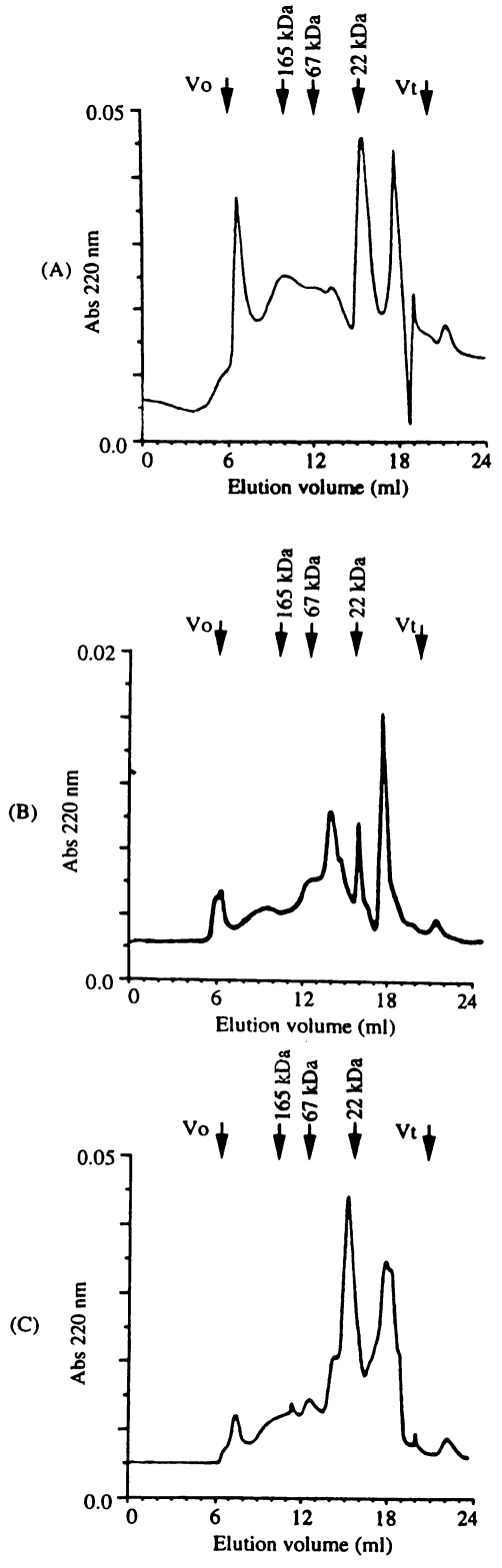


Fig. 20: Gel permeation chromatography of the Sephadex void volume peaks resulting from the successive chromatography of the HMWcitr from Fig. 16 on the Sephadex G25, G15 and G10.
a) HMW_{G25Vo},
b) HMW_{G15Vo},
c) HMW_{G10Vo}
Column: analytical Superose 12 HR10/30.
Sample: 250 µl of a, b, or c mixed 1:1 with 0.2 mol/l Tris-HCl, at pH 7.0
Eluent: 0.1 mol/l Tris- HCl buffer, at pH 7.0
Flow rate: 0.5 ml/min

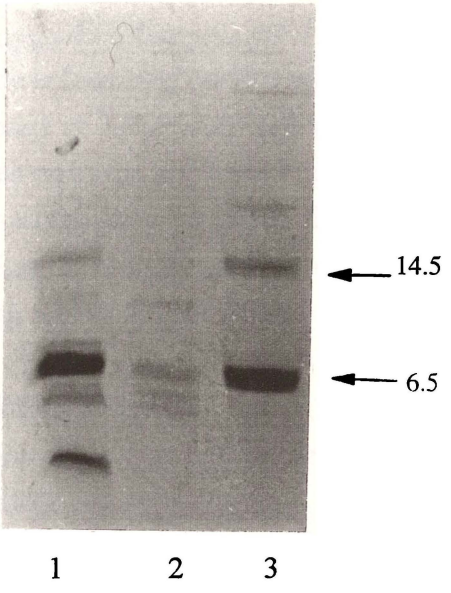


Fig. 21: SDS-PAGE under reducing conditions of the material in the void volume peaks resulting from the chromatography of HMWcitr on the Sephadex columns-
Lane 1: HMW_{G25Vo}. Lane 2: $HMW_{G15\ Vo}$. Lane 3: HMW_{G10Vo}.

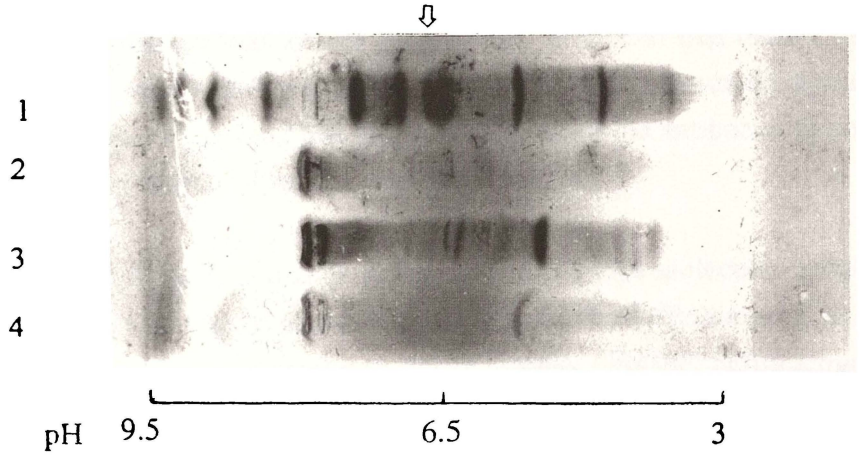


Fig. 22: Isoelectric focussing of the void volume peak from the chromatography on the Sephadex columns.
Lane 1: standards pI 3 to 9.5. Lane 2: HMW_{G15Vo}. Lane 3: HMW_{G25Vo}. Lane 4: HMW_{G10Vo}.
⇨ shows point of sample application

Isoelectric focussing

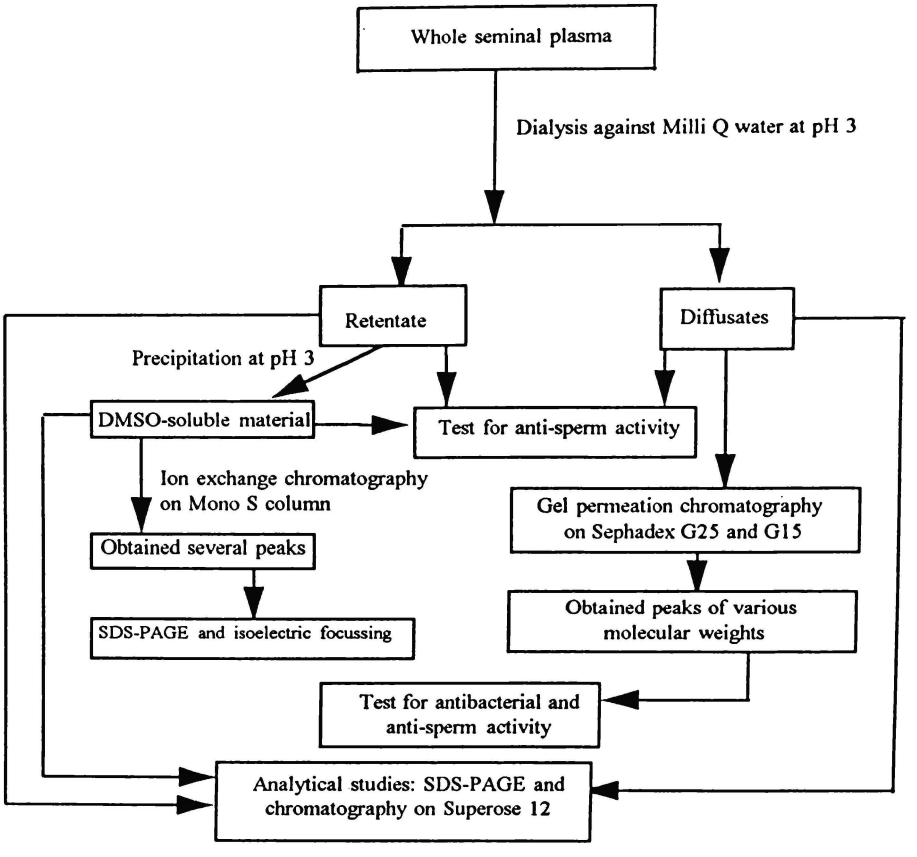
SDS-PAGE and gel permeation chromatography of HMW_{G25Vo}, $HMW_{G15\ Vo}$ and HMW_{G10Vo} fractions indicated a diversity of molecular weights. Cationic proteins have been implicated in the anti-sperm (Shannon *et al.*, 1974; Baas, 1985) and antibacterial activity (Shannon *et al.*, 1987; Hameed, 1987). To determine the charge of the components in HMW_{G25Vo}, $HMW_{G15\ Vo}$ and HMW_{G10Vo}, a sample of the peaks was subjected to isoelectric focussing performed on a PhastSystem using a PhastGel IEF gel with a pH range of 3 to 9 as described in Chapter 2. The gel was stained by silver staining. The results are shown in Fig. 22. Several bands were focussed in both the cationic and the anionic region. The G10 void volume peak contained components that migrated toward the cationic area, one of which focussed at the edge of the gel indicating a pI of 9.5 or higher.

3.3.3.2 SUMMARY AND CONCLUSION:

Gel permeation chromatography of the retentate of seminal plasma dialysed against citrate buffer with citrate as a dissociating agent (Fig. 16) revealed that proteins in the retentate have molecular weights ranging between 300 kDa and 1 kDa (the separation range of the preparative Superose 12 column). Anti-sperm activity was found over the whole range but was significantly greater in the fraction of lowest molecular weight obtained (LMWcitr). Although no inhibitory effect on sperm motility was seen soon after addition of this fraction, the effect was quite marked after 18h incubation at ambient temperature. The antibacterial activity was also found over the whole range but was lowest in LMWcitr. It is clear that the antibacterial and anti-sperm activities are due to different components in seminal plasma. The scope of this thesis does not extend into the investigation of the antibacterial component, hence any further antibacterial testing was not attempted.

Dialysis of the fractions from Fig. 16 in a 3.5 kDa molecular weight cut-off dialysis membrane produced material in the diffusate from fractions of high and low molecular weight that are detrimental to sperm motility. Further chromatography of the fractions from Fig. 16 (*i.e.* HMWcitr, MMWcitr and LMWcitr) under acidic conditions, yielded a low molecular weight fraction estimated to be between 5 and 1 kDa (*i.e.* the exclusion limit of the Sephadex G15 and G10 columns). This fraction showed marked anti-sperm activity. The fractions differed in the extent of their activity but in general an increase in anti-sperm activity was seen after dissociation of the high molecular weight aggregates with citrate or acid.

Flow chart for Section 3.4



Components in HMW_{G25V_0},.$HMW_{G15\ V_0}$ and HMW_{G10V_0}, although having an apparent molecular weight of less than 5 kDa, aggregated to a higher molecular weight. SDS-PAGE and isoelectric focussing of these fractions showed the diversity of molecular weight (between 2 and 20 kDa) and charges (cationic and anionic).

3.4 ATTEMPTS TO DISSOCIATE THE ANTI-SPERM COMPONENT FROM THE AGGREGATE BY DIALYSIS OF SEMINAL PLASMA AT pH 3

In Section 3.3, dialysis against citrate buffer was shown to result in the release of low molecular weight active components. However, the activity also remained associated with the high molecular weight fraction. Chromatography of the high molecular weight fraction under acidic conditions resulted in further release of the low molecular weight active components. Thus, acidification may be a useful tool to dissociate the active peptide. Shannon *et al.* (1987) reported that less than 12.5% of the antibacterial activity was retained in the dialysis membrane after exhaustive dialysis under acidic conditions. Thus, extensive dialysis could result in the active components passing through the dialysis membrane, and the activity of the retentate would be markedly reduced. Accordingly, dialysis of seminal plasma under acidic conditions was conducted. It was assumed that the proteins inside the dialysis membrane would disaggregate under the acidic conditions. Components of molecular weight less than the retentivity of the membrane (3.5 kDa) will pass through the membrane and will appear in the diffusate. The proteins that are larger than the cut-off point of the membrane will stay inside the dialysis membrane.

Seminal plasma was obtained from fresh semen by centrifugation at 20 000 *g* and filtration through a 0.2 μm membrane. From this, 10 ml was adjusted to pH 3 with HCl and dialysed against 1 L of water acidified with HCl (at pH 3) in a dialysis membrane (19 mm flat width) of 3.5 kDa molecular weight cut-off. The dialysis was carried out for 72 h at 4°C with continuous stirring and the diffusate was removed and replaced by 1 L of acidified water (at pH 3) every 18 h.

The pH of each diffusate was adjusted to 7 and the volume reduced to 5 ml (half the original volume of seminal plasma) by rotary evaporation. The concentrates were desalted on a column of Sephadex G10 eluted with water and the desalted material was concentrated back to 5 ml by rotary evaporation. They were then retained separately to be tested for anti-sperm activity and further studies. The diffusates were designated 1AD (first acid diffusate), 2AD (second acid diffusate), 3AD (third acid diffusate) and 4AD (fourth acid diffusate).

After the completion of dialysis, the retentate appeared to have some sedimentation. This sediment was separated by centrifugation at 20 000 *g* for 30 min and the clear supernatant was adjusted to pH 7, the volume reduced by rotary evaporation to 5 ml (half the original volume of seminal plasma) and retained frozen for further studies. To the insoluble material a few drops of dimethyl sulphoxide was added and it was resuspended in 5 ml of water. This was stored frozen for further studies and designated as DMSO-soluble material.

3.4.1 PRELIMINARY INVESTIGATION FOR ANTI SPERM-ACTIVITY IN THE DIFFUSATES AND RETENTATE OF SEMINAL PLASMA THAT WAS DIALYSED AT pH 3

3.4.1.1 PREPARATION OF THE SAMPLES

A 1 ml sample of the concentrated retentate, the DMSO-soluble material and each of the concentrated diffusate from the acid dialysis was diluted with 1 ml DS14G diluent for testing anti-sperm activity. A 1 ml sample of seminal plasma was adjusted to pH 3, incubated for 18 h and then the pH was adjusted to 7. This treated seminal plasma was included for comparison. The acid may affect the status of the protein aggregates and pre-treatment of seminal plasma with acid would then show any changes in the activity compared with the retentate of dialysed seminal plasma which was not treated.

3.4.1.2 TEST FOR ANTI-SPERM ACTIVITY

From the prepared samples mentioned above, a 100 µl sample was added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of $10\text{-}12 \times 10^6$ sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 14.

The results (shown in Table 14) indicated that all the diffusates reduced the percentage of motile sperm after 18 h incubation at ambient temperature, however, the highest activity was in the first diffusate. The retentate and the DMSO-soluble material, after exhaustive dialysis under acidic conditions, also retained high anti-sperm activity, and the reduction in the percentage of the motile sperm was significant ($p<0.05$).

Table 14. Effect of the acid diffusate and retentate of seminal plasma on the motility of washed bull sperm

The values for motility shown are the percentage of sperm motile immediately after addition and after 18 h incubation at ambient temperature. Values are mean ± s.e.m. (n=4). All fractions were at 10% of their original concentration in seminal plasma. Acid treated DSP is the retentate of dialysed seminal plasma its pH reduced to 3 then neutralised.

Treatment	Protein conc. mg/ml	% Motile	
		0 h	18 h
Control (diluent)		82.5±1.4^{a}	72.5±1.7^{a}
1st diffusate (1AD)	1.20	72.5±1.6^{bc}	47.5±2.0^{d}
2nd diffusate (2AD)	0.65	72.5±1.6^{bc}	57.5±2.0^{c}
3rd diffusate (3AD)	0.43	72.5±1.6^{bc}	56.3±2.4^{c}
4th diffusate (4AD)	0.32	75.0±2.0^{b}	56.3±4.3^{c}
Retentate	1.19	71.3±1.3^{c}	52.5±4.3^{cd}
DMSO soluble material	0.28	71.7±1.3^{c}	47.5±2.0^{d}
Acid treated seminal plasma	4.13	76.3±1.2^{b}	58.7±1.3^{c}
DSP*	3.63	82.5±1.5^{a}	64.3±1.8^{b}

**Retentate of seminal plasma against dialysed water at 10% concentration for comparison.*

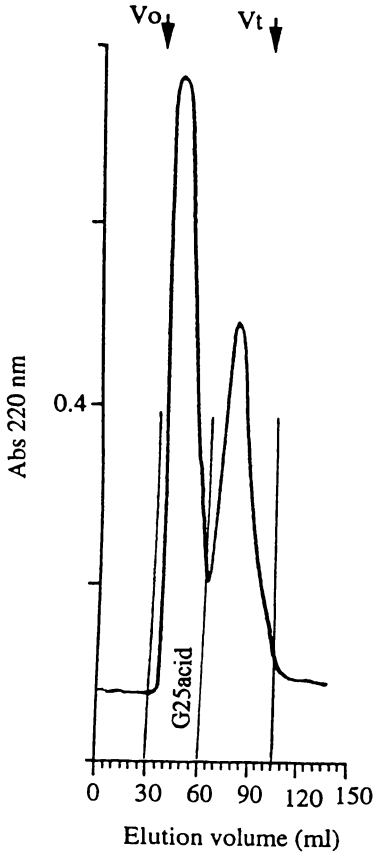
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The retentate of seminal plasma dialysed at pH 3 was significantly more damaging to sperm than the retentate of seminal plasma dialysed against water. This increase in the total activity following the removal of diffusible components, which are detrimental to sperm, indicates disaggregation and formation of a new equilibrium between the high and the low molecular weight components (which may be responsible for the higher activity). Interestingly, the anti-sperm activity had increased after the treatment with acid without dialysis.

3.4.2 PROCESSING THE DIFFUSATE FROM DIALYSIS OF SEMINAL PLASMA AT pH 3

3.4.2.1 GEL PERMEATION CHROMATOGRAPHY ON SEPHADEX

The pH of 4 ml of the concentrated first diffusate from acid dialysis was adjusted to 1.7, and a 2 ml sample chromatographed on a column of Sephadex G25SF eluted with 0.02 mol/l HCl, pH 1.7 as detailed in Fig. 23. There was a large peak at the void volume followed by another peak in the region of molecular weight less than 5 kDa. The chromatography was repeated. The fractions comprising the void volume peak (fractions 30 to 60 ml) from each run were pooled and the pH adjusted to 7, then they



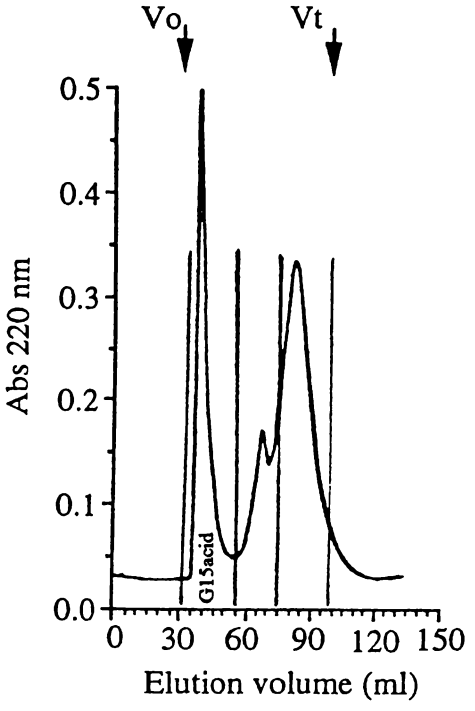


Fig. 23

Fig. 24

Fig. 23: Gel permeation chromatography of the diffusate of seminal plasma dialysed under acidic conditions (at pH 3).
Gel: Sephadex G25 SF
Sample: 2 ml of the concentrated material adjusted to pH 1.7
Eluent: 0.02 mol/l HCl, pH 1.7
Fraction volume: 5 ml
Flow rate: 1 ml/min

Fig. 24: Gel permeation chromatography of the material in the second peak in Fig. 23.
Gel: Sephadex G15
Sample: 2 ml of the concentrated material adjusted to pH 1.7
Eluent: 0.02 mol/l HCl, pH 1.7
Fraction volume: 5 ml
Flow rate: 1 ml/min

were rotary evaporated to 2 ml (half the original volume). This fraction was designated G25acid.

The second peak from Sephadex G25 in Fig. 23 was further processed. The fractions comprising this peak were pooled and rotary evaporated to 2 ml. The concentrate was centrifuged at 20 000 *g* for 10 min then chromatographed on a column of Sephadex G15 eluted with 0.02 mol/l HCl as detailed in Fig. 24. This material was seen to contain components that eluted at the void volume of the column of Sephadex G15, with estimated molecular weights of 1.5 kDa or more, followed by another two unresolved peaks with estimated molecular weights less than 1.5 kDa. The fractions comprising the void volume peak were pooled, the pH adjusted to 7, and reduced to 2 ml (half the original volume) by rotary evaporation. This fraction was designated G15acid. Fractions from 55 to 70 ml of the second peak in Fig. 24 were collected, the pH was adjusted to 7 and the volume reduced by rotary evaporation to 2 ml (half the original volume). The fractions comprising the bed volume peak (70 to 100 ml) were collected, the pH adjusted to 7, and reduced to 2 ml (half the original volume) by rotary evaporation. The fractions comprising the peaks in Figs. 23 and 24 were desalted on Sephadex G10 eluted with water and the desalted material was concentrated back to 2 ml to be tested for anti-sperm activity. The protein concentrations were determined and 1 ml of each was diluted 1:1 with DS14G diluent for testing anti-sperm activity.

Testing for anti-sperm activity in the peaks from the chromatography of the acid diffusate

From each of the prepared samples a 100 µl aliquot was added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C).

The results (in Table 15) show a marked effect of all the fractions from G25 and G15 on sperm motility after incubation for 18 h. Addition of the G25acid from Fig. 23 and the fractions 70 to 100 ml from Fig. 24 immediately reduced the percentage of motile sperm as compared with the control.

Table 15. Effect of the fractions from Figures 23 and 24 on the motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=4). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein Conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		82.5±1.4^{a}	72.5±1.5^{a}
G25acid	0.68	71.3±2.4^{b}	50.0±2.0^{c}
G15acid	0.15	81.3±1.3^{a}	46.3±3.7^{c}
Fractions 55-70 ml from Fig. 24	0.08	81.3±1.3^{a}	65.0±2.4^{b}
Fractions 70-100 ml from Fig. 24	0.27	65.0±2.4^{c}	37.5±1.4^{d}
DSP*	3.63	81.3±1.3^{a}	63.8±1.2^{b}

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison.*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

3.4.3 ANALYTICAL STUDY OF THE DIFFUSATES AND THE RETENTATE FROM ACID DIALYSIS OF SEMINAL PLASMA

The preliminary investigation of the acid diffusates showed that anti-sperm activity passed through the dialysis membrane of 3.5 kDa molecular weight cut-off. The activity passed through after several changes indicating a continuous dissociation from the high molecular weight component. Dissociation of the low molecular weight component(s) from the macromolecule under acidic conditions did not diminish the activity in the retentate indicating that the activity was still present in the components with molecular weight higher than 3.5 kDa.

Gel permeation chromatography under neutral conditions and SDS-PAGE under reducing conditions was therefore conducted to determine the composition and the molecular weight of the components in the diffusate and the retentate of seminal plasma dialysed at pH 3.

3.4.3.1 GEL PERMEATION CHROMATOGRAPHY OF THE ACID DIFFUSATE UNDER NEUTRAL CONDITIONS

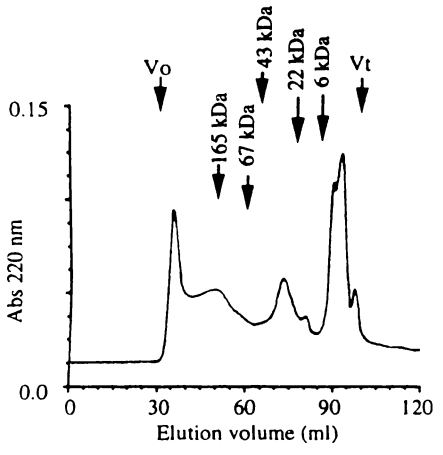
The material that passed through the dialysis membrane under acidic conditions presumably has a molecular weight of 3.5 kDa or less. To determine if this material in the diffusate had the ability to form aggregates of higher molecular weight, a 500 µl

sample from the concentrated first acid diffusate (1AD) was mixed 1:1 with 0.2 mol/l Tris-HCl, at pH 7, and incubated for 2 h at 37°C. After incubation it was centrifuged at 20 000 *g* for 10 min then chromatographed on the preparative Superose 12 column eluted with 0.1 mol/l Tris-HCl at pH 7. The elution profile (in Fig. 25) shows that the material in the diffusates, which passed through a membrane of 3.5 kDa molecular weight cut off, reaggregated and a void volume peak with estimated molecular weight of 300 kDa or higher was obtained. However, a large portion of the protein eluted at the relatively low molecular weight region (less than 20 kDa).

3.4.3.2 GEL PERMEATION CHROMATOGRAPHY UNDER NEUTRAL AND ACIDIC CONDITIONS OF THE RETENTATE OF SEMINAL PLASMA DIALYSED AT pH 3

The material retained in the dialysis membrane after dialysis of seminal plasma at pH 3 should have been of molecular weight greater than 3.5 kDa. To determine if the material in the retentate had been affected by the acid conditions and still had the ability to form aggregates, a 500 µl aliquot of the retentate was mixed 1:1 with 0.2 mol/l Tris-HCl, at pH 7, and incubated for 2 h at 37°C. After incubation it was centrifuged at 20 000 *g* for 10 min then chromatographed on the preparative Superose 12 column eluted with 0.1 mol/l Tris-HCl at pH 7. The elution profile (in Fig. 26) shows that the material in the retentate was still in an aggregated form and a peak at the void volume appeared, however, a reasonable amount of the protein eluted at the lower molecular weight region (see Figs. 1 and 2 for comparisons with the retentate of dialysis against water).

To determine the molecular weight of the components in the retentate under acidic conditions, the retentate was subjected to chromatography at pH 3. Accordingly, a 500 µl sample of the retentate at pH 3 was incubated for 2 h at 37°C. After incubation it was centrifuged at 20 000 *g* for 10 min, then chromatographed on the preparative Superose 12 column and eluted with dilute HCl at pH 3. The elution profile (in Fig. 27) showed a major shift towards the low molecular weight region. The profile showed a major peak not resolved from other smaller peaks and a small peak at the area after the bed volume of the Superose 12 column. The large peak covered the area from 55 to 86 ml with estimated molecular weight of between 6 to 70 kDa and probably consists of the bulk of the stable aggregates in bovine seminal plasma. The appearance of a sharp peak with an estimated molecular weight of less than 5 kDa at the bed volume indicates that partial disaggregation has occurred.



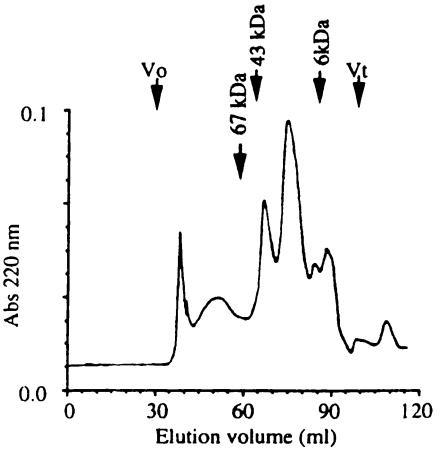


Fig. 25 **Fig. 26**

Fig. 25: Gel permeation chromatography of the diffusate resulting from dialysis of seminal plasma under acidic conditions.
Column: preparative Superose 12.
Sample: 500 µl of the concentrated acid diffusate mixed 1:1 with 0.2 mol/l Tris -HCl, pH 7.0
Eluent: 0.1 mol/l Tris- HCl buffer, pH 7.0.
Flow rate: 1 ml/min

Fig. 26: Gel permeation chromatography of the retentate resulting from dialysis of seminal plasma under acidic conditions.
Column: preparative Superose 12.
Sample: 500 µl of the retentate mixed 1:1 with 0.2 mol/l Tris -HCl, pH 7.0
Eluent: 0.1 mol/l Tris- HCl buffer, pH 7.0.
Flow rate: 1 ml/min.

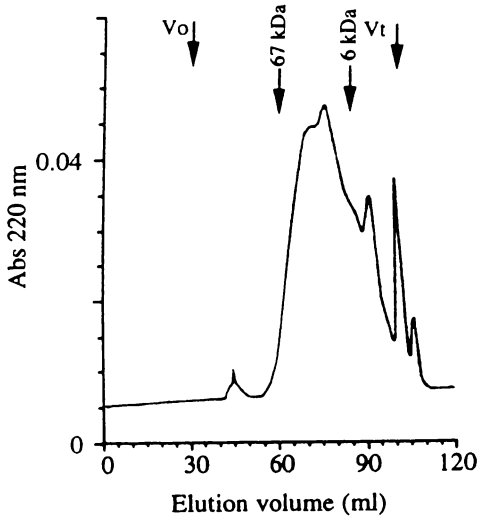


Fig. 27: Gel permeation chromatography of the retentate resulting from dialysis of seminal plasma under acidic conditions.
Column: preparative Superose 12.
Sample: 500 µl of the retentate at pH 3.0.
Eluent: water-HCl, pH 3.0.
Flow rate: 1 ml/min

Clearly, acidic conditions affect the status of the large molecular weight components in seminal plasma, however, complete disaggregation did not take place.

3.4.3.3 SDS-PAGE OF THE ACID DIFFUSATE AND THE RETENTATE OF SEMINAL PLASMA DIALYSED AT pH 3

SDS-PAGE under reducing conditions was conducted to determine the molecular weight of the components that comprised the diffusate obtained from the dialysis of seminal plasma under acidic conditions. Samples from the concentrate of the first and the fourth diffusate were prepared as described in Chapter Two and 10 µl was loaded onto a 20% polyacrylamide gel. The acid diffusate contained a large number of bands (see Fig. 28) with molecular weights up to 14 kDa. It was interesting to find bands of higher molecular weight than the retentivity of the dialysis membrane (*i.e.* 3.5 kDa) even in the presence of SDS. The appearance of bands with molecular weights of up to 14 kDa is evidence that the smaller components of less than 3.5 kDa had aggregated. It was of interest that the diffusate of seminal plasma dialysed against citrate also showed a similar pattern (Fig. 15). In both cases dissociation was achieved by interfering with the ionic attraction of the molecules. These common components may be formed by other forces such as the hydrophobic attraction. Very low molecular weight components were still present in the diffusate as can be seen in Fig. 28.

The retentate of seminal plasma dialysed at pH 3 was also subjected to SDS-PAGE under reducing conditions. Samples were prepared as described in Chapter Two and a 10 µl sample was loaded onto a 12.5% polyacrylamide gel. The results are shown in Fig. 29. The retentate showed the presence of bands common to seminal plasma, however, some bands did not stain to the same intensity or were not present. Nevertheless, bands at the high molecular weight region were present and as seen previously: the 14 to 18 kDa bands were prominent.

3.4.4 ANALYTICAL STUDY OF THE DIMETHYL SULPHOXIDE-SOLUBLE PRECIPITATE FROM ACID DIALYSIS OF SEMINAL PLASMA

3.4.4.1 ANALYTICAL GEL PERMEATION CHROMATOGRAPHY OF THE DMSO-SOLUBLE PRECIPITATE

The precipitate obtained after dialysis of seminal plasma at pH 3 that dissolved with the addition of dimethyl sulphoxide (DMSO-soluble material) exhibited high anti-sperm activity (Section 3.4, Table 14). It was of interest to determine the composition of this material. Accordingly the DMSO-soluble material (prepared as

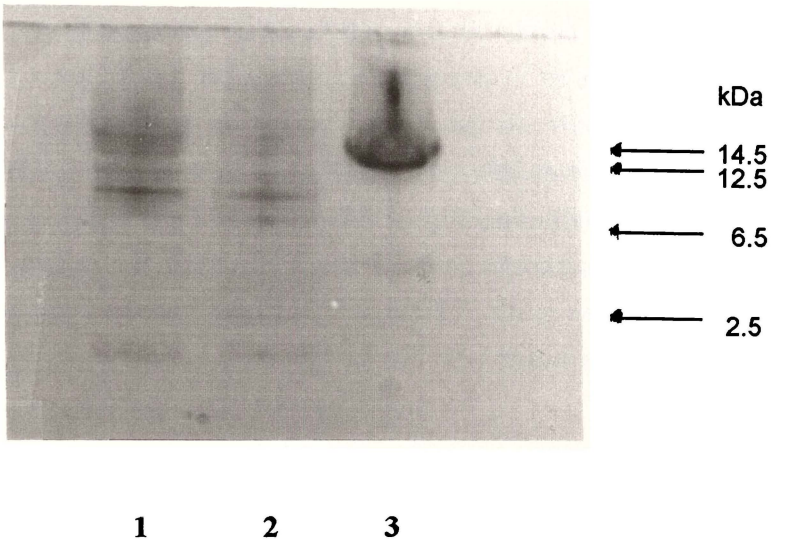


Fig. 28: SDS-PAGE of the material appearing in the diffusate resulting from the dialysis at pH 3 of seminal plasma in dialysis membrane with 3.5 kDa molecular weight cut-off. A 20% polyacrylamide gel was used under reducing conditions.
Lane 1: 1AD. Lane 2: 4AD. Lane 3: lysozyme (14.5 kDa).

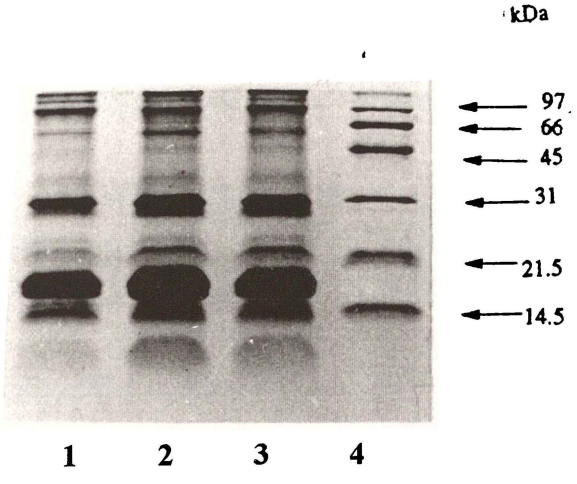


Fig. 29: SDS-PAGE under reducing conditions of the retentate of the retentate of dialysed seminal plasma against acid on a 12.5% polyacrylamide gel.
Lane 1: the retentate of dialysed seminal plasma against water pH 3. Lane 2 and 3: whole seminal plasma. Lane 4: molecular weight markers.

described in Section 3.4.1) was centrifuged at 20 000 g for 10 min and a 250 µl aliquot of the supernatant was subjected to gel permeation chromatography on the analytical Superose 12 column eluted with 0.1 mol/l Tris-HCl buffer, pH 7. The results (shown in Fig. 30) indicate that most of the material eluted at the area near the bed volume, between 14 and 20 ml, indicating a component of relatively low molecular weight (less than 40 kDa), however, a peak near the void volume was obtained indicative of the presence of an aggregate. This chromatography showed a good separation of the high and the low molecular weight components. When the pH of the sample was reduced to 1.7, held at 4°C for 2 h then chromatographed on the Superose 12 column eluted with 0.02 mol/l HCl at pH 1.7, there was a shift to the lower molecular weight region between 17 and 20 ml. The material was resolved into three peaks, one of them at the bed volume (Fig. 31).

3.4.4.2 SDS-PAGE OF THE DMSO-SOLUBLE MATERIAL

SDS-PAGE was performed on the DMSO-soluble material using a 20% polyacrylamide gel under reducing conditions. The sample was prepared as described in Chapter Two and 10 µl was loaded onto the gel. The result (shown in Fig. 32) indicated that the DMSO-soluble material contained components of relatively low molecular weight. Several bands were seen between 12 and 14 kDa and a trace of bands in the high molecular weight region was also observed. Similar results were obtained for the precipitate of the retentate of dialysed seminal plasma as seen in Fig. 7.

3.4.4.3 ION EXCHANGE CHROMATOGRAPHY OF THE DMSO-SOLUBLE MATERIAL

The DMSO soluble material was further processed by anion exchange chromatography. This procedure was to determine whether separation by charge would release the active anti-sperm component. Accordingly, more of the precipitate was obtained as described before. A 1 ml sample of the concentrate was mixed 1:1 with 50 mmol/l Tris-HCl-buffer, pH 8 and loaded onto a Mono Q column (anion exchanger) eluted with a gradient as detailed in Fig. 33. pH 8 was chosen because the basic components in seminal plasma were expected to be positively charged at this pH since the 20 kDa component migrated ahead of lysozyme (Hameed, 1987) and the aggregates in bovine seminal plasma behave as if basic in nature (P. Molan, personal communication). The results in Fig. 33 show that most of the material bound to the column and was eluted in several poorly resolved peaks. The fractions comprising the peaks 32 to 34 min, 34 to 36 min, 36 to 38 min, 38 to 40 min, 40 to 44 min and 44 to 48 min were pooled separately.

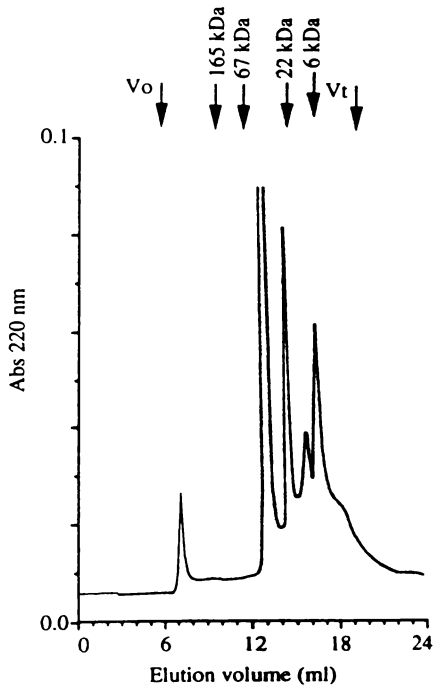


Fig. 30

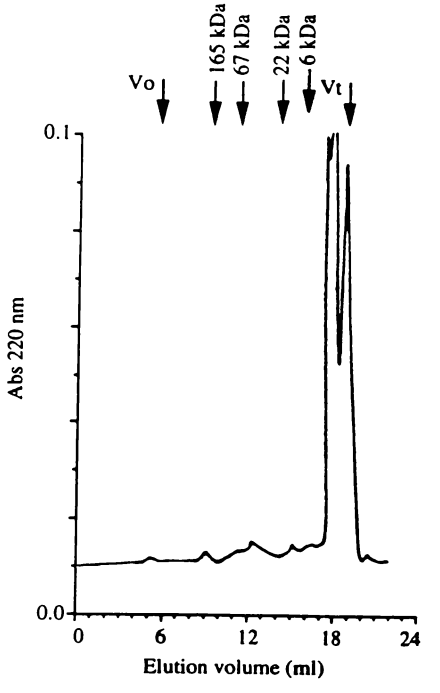


Fig. 31

Fig. 30: Gel permeation chromatography of the DMSO-soluble material.
Column: analytical Superose 12.
Sample: 250 µl of the DMSO soluble material at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 0.5 ml/min

Fig. 31: Gel permeation chromatography of the DMSO-soluble material.
Column: analytical Superose 12.
Sample: 250 µl of the DMSO soluble material at pH 1.7.
Eluent: 0.02 mol/l HCl , pH 1 7.
Flow rate: 0.5 ml/min

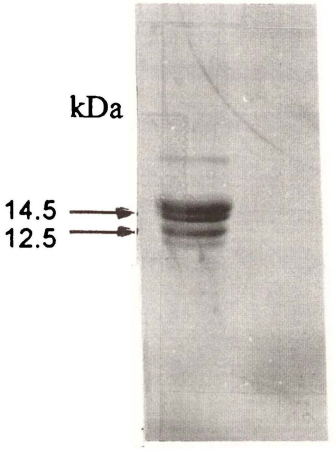


Fig. 32: SDS-PAGE under reducing conditions of the DMSO-soluble material. A 10 µl sample was loaded on a 20% polyacrylamide gel.
Lane 1: DMSO-soluble material. Lane 3: cytochrome c (12.5 kDa).

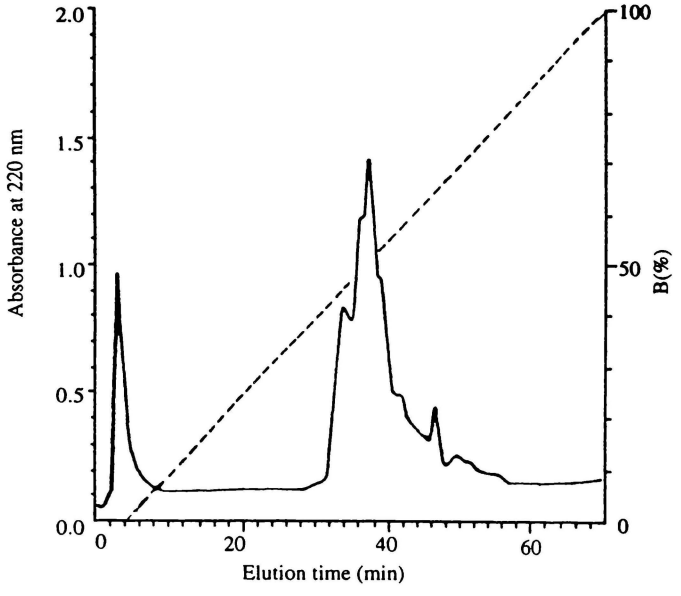


Fig 33: Chromatography of DMSO-soluble material on an anion exchanger.
Column: Mono Q HR 5/10
Sample: 2 ml of the DMSO soluble material in 25 mmol/l Tris-HCl buffer at pH 8.
Eluent A: 25 mmol/l Tris-HCl, pH 8.
Eluent B: 2 mol/l NaCl in 25 mmol/l Tris-HCl, pH 6.5.
Gradient: 0-100% B in 64 min.
Flow rate: 1 ml/min.

The fractions comprising the unbound material (2 to 4 min) were also pooled. All fractions were desalted on a Sephadex G10 column (Pharmacia XK 10/20).

SDS-PAGE of fractions from the chromatography shown in Fig. 33

To determine the molecular weight of the separated components from ion exchange chromatography, SDS-PAGE was conducted under reducing conditions on a 20% polyacrylamide gel. Samples were prepared as before and 10 µl was loaded on the gel. All fractions contained a major common band appearing as a doublet with other bands of lower and higher molecular weights (Fig. 34). The major peak 36 to 38 min appeared to have a complex of molecules similar to those seen in Fig. 32 and Fig. 7. The ion exchange procedure obviously did not disaggregate the larger components since the unbound material also appeared to have fractions with similar molecular weights.

Isoelectric focussing of fractions from Fig. 33

Anion exchange chromatography revealed that there were components which were not bound to the column at pH 8 indicating different charges of the molecules. SDS-PAGE revealed that all the fractions from the anion exchanger have a common component even though they were eluted with different salt concentrations. To gain a better idea about the charge of these components, it was decided to determine their isoelectric points. This would also give some idea about the composition of these components which appeared to have similar molecular weights. Accordingly, an isoelectric focussing experiment was performed on a Pharmacia PhastSystem using pre-cast gel with a pH range of 3 to 9. The results are shown in Fig. 35. The fractions were seen to consist of a complex of components migrating to both the cationic and the anionic end. Several bands were observed on the gel with each fraction.

3.4.5 SUMMARY AND CONCLUSION:

The diffusate resulting from dialysis of seminal plasma under acidic conditions contained low molecular weight components which had a detrimental effect on sperm motility. SDS-PAGE under reducing conditions showed that the diffusate was composed of a complex of low molecular weight components. It was evident that disaggregation had occurred, however, these components reaggregated to a larger molecular weight complex even in the presence of SDS. Gel permeation chromatography also showed reaggregation to higher molecular weight components.

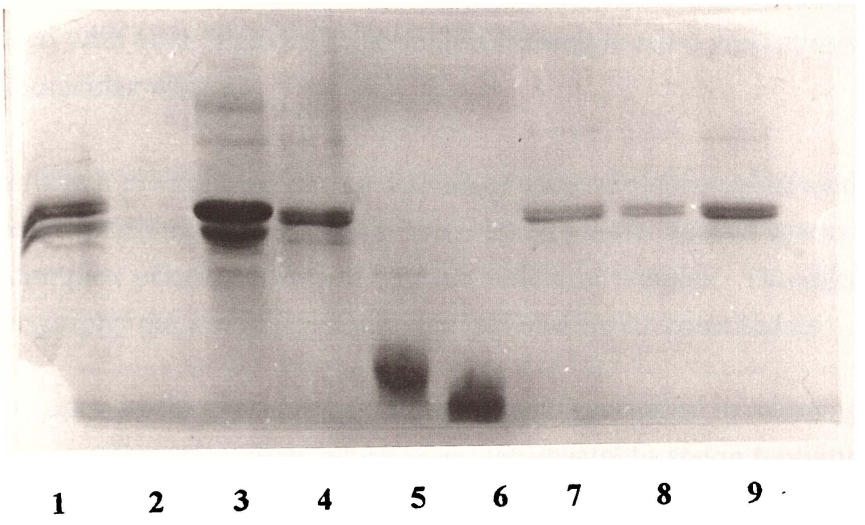


Fig. 34: SDS-PAGE of the fraction from Fig. 33 under reducing conditions. A 10 µl sample was loaded on a 20% polyacrylamide gel.
Lane 1: fractions 2 to 4 min. Lane 2: fractions 44 to 48 min. Lane 3: fractions 36 to 38 min. Lane 4: 38 to 40 min. Lane 5: insulin B chain (3.4 kDa). Lane 6: bacitracin (1.45 kDa). Lane 7: fractions 32 to 34 min. Lane 8: fractions 40 to 44. Lane 9: 36 to 38 min

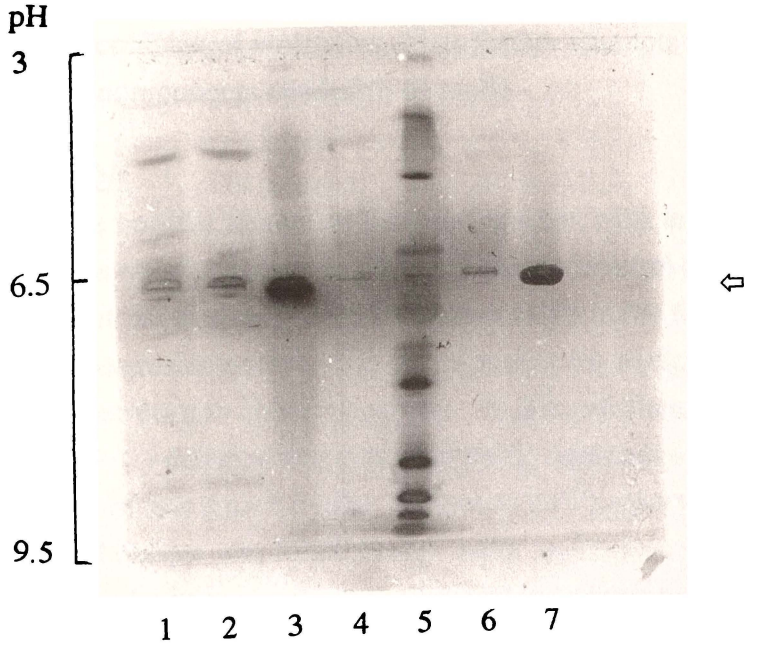


Fig. 35: Isoelectric focussing of fractions in Fig. 33.
Lane 1: fractions 2 to 4 min. Lane 2: 36 to 38 min. Lane 3: fractions 38 to 40 min. Lane 4: fractions 32 to 34 min. Lane 5: standard (pI 3 to 9.5). Lane 6: fractions 40 to 44. Lane 7: fractions 44 to 48 min.
⇨ shows point of sample application

The retentate from dialysis of seminal plasma under acidic conditions contained a complex of components of varying molecular weights as shown by SDS-PAGE. Upon gel permeation chromatography under neutral conditions, the retentate generally appeared to have a similar elution profile to that observed in seminal plasma not treated with acid. Peaks of high molecular weight were present as well as some of low molecular weight.

Material which precipitated during dialysis of seminal plasma under acidic conditions, solublised with dimethyl sulphoxide, had marked activity against sperm and appears to be a complex of components of various molecular weights. The anion exchange chromatography did not disaggregate the molecule to its constituents.

Disaggregation in the presence of citrate and acid succeeded in releasing low molecular weight components which were detrimental to sperm motility although part of the activity was still present in the high molecular weight components. It was concluded that dialysis against citrate buffer and against water at pH 3 and the subsequent gel permeation chromatography under acidic condition partially dissociated low molecular weight components which are detrimental to sperm motility, however, these low molecular weight components favour forming aggregates of higher molecular weight which also are detrimental to sperm motility. Treatment with acid did not completely dissociate the activity as all the fractions appeared to be a complex of molecules. Thus further attempts to dissociate and isolate the active components needed to be made.

3.5 DISCUSSION

Proteins in bovine seminal plasma, in their native state, exist in a wide range of molecular weights ranging from 5 to 500 kDa. Under neutral conditions, it appears that a large part of the protein in seminal plasma exists in the form of high molecular weights aggregates as demonstrated in the present study using dialysis experiments followed by gel permeation chromatography. Studies by Shannon *et al.* (1987) and Hameed *et al.* (1991) describe this system as being aggregates of small units of low molecular weight of less than 3.5 kDa. Hameed (1987) reported that the bulk of the protein in seminal plasma was found in two major aggregates with estimated molecular weight of 500 and 250 kDa. These large aggregates possess antibacterial activity, and have been described as a complex made up of small units of molecular weights ranging between 1-3.5 kDa (Shannon *et al.*, 1974;1987; Eschenbruch, 1980; Hameed, 1987). This was confirmed in the present study.

The aggregates of high molecular weight, under disaggregating conditions such as low pH or in the presence of citrate at pH 7, release low molecular weight components which in turn re-aggregate to the higher molecular weight components when chromatographed once the disaggregation conditions are removed. It was proposed that these aggregates are held by ionic attraction and hydrophobic clustering (Hameed *et al.*, 1991).

The anti-sperm and the antibacterial activity have both been attributed to the protein(s), or peptides present in bovine seminal plasma. Shannon *et al.* (1974) observed that the antibacterial and the anti-sperm activity were present in all fractions obtained from seminal plasma. They proposed that the two activities may be due to the same components. Gel permeation chromatography of seminal plasma pre-dialysed against water at neutral conditions (Fig. 1) showed that antibacterial and anti-sperm activity (Tables 2 and 3) was distributed over a wide range of molecular weight components. However, the anti-sperm activity was more prominent in the components with molecular weights less than 20 kDa, while the antibacterial activity was more pronounced in the proteins in an aggregated state of molecular weight higher than 20 kDa. In fact the antibacterial activity was at its highest in whole seminal plasma (Table 3). The components responsible for these two activities seem to differ in molecular size.

The antibacterial activity in seminal plasma has been extensively investigated (Hameed, 1987; Shannon *et al.*, 1987). It was confirmed by Hameed (1987) that the 500 kDa aggregate contains the highest proportion of the antibacterial activity while the 20 kDa aggregate had the lowest proportion of the antibacterial activity. In this study, dialysed seminal plasma and the high molecular weigh aggregates over 20 and up to 300 kDa had high antibacterial activity. In contrast, the anti-sperm activity was pronounced in the low molecular weight component(s) of less than 20 kDa. Further disaggregation of dialysed seminal plasma using citrate produced low molecular weight component(s) with anti-sperm activity much higher than dialysed seminal plasma itself. Thus, aggregation in the form of high molecular weight components may serve two activities: protecting the male reproduction tract against bacterial invasion and protect the sperm from the toxic effect of the low molecular weight components.

Dialysis for 48 hours was conducted to remove all of the small molecules and the ions in the seminal plasma with molecular weight less than the retention capacity of the dialysis membrane (*i.e* <12 kDa). Precipitation of the protein in seminal plasma

resulted from this, possibly because the diffusion of citrate, other ions and small molecules, present in the native seminal plasma, may have restored the ionic strength allowing most of the proteins back into solution. The addition of ions causing re-solubilisation of the precipitate does not rule out the possibility that conformational changes may have taken place (*e.g.* strong hydrophobic interaction) accounting for the small amount of precipitate that did not go back into solution.

When seminal plasma was dialysed against Milli Q water under neutral conditions, using a 12 kDa cut-off dialysis membrane, the retentate retained antibacterial as well as the anti-sperm activity as shown in Table 1 and Table 2 respectively. Repeated dialysis of the pre-dialysed seminal plasma against water under neutral conditions showed that neither antibacterial nor anti-sperm activity could be detected in further diffusates. This indicates that the anti-sperm and the antibacterial activity under neutral conditions, at least in part, is still associated with components of greater molecular size than the membrane's cut-off of 12 kDa . However, part of the anti-sperm and to a lesser extent, antibacterial activity passed through the dialysis membrane and was observed in the first diffusate of seminal plasma dialysed against water. The primary screening for the anti-sperm activity in the retentate of seminal plasma dialysed against water revealed that the low molecular weight fractions possess the highest anti-sperm activity. This observation is in agreement with Collingwood (1990) who reported that the fraction <20 kDa was the most active fraction and very detrimental to sperm survival.

The process of aggregation was evident. When the peak with estimated molecular weight 300 kDa was subjected to SDS-PAGE under reducing conditions, bands of varying molecular weight appeared between 200 kDa (or greater) down to 10 kDa. The intensity of the bands, however, was more prominent in the region less than 20 kDa. The presence of SDS may have affected the status of the aggregates through disruption of the ionic interactions, thus causing disaggregation.

SDS-PAGE under reducing and non-reducing conditions revealed the presence of more than 10 bands stained for protein. The reducing condition did not affect the profile indicating that the disulphide bonding is not a contributor to the aggregation system, an observation in agreement with that of Eschenbruch (1980) and Hameed (1987). In contrast to the gel permeation chromatography elution profile, the major protein(s) was in the relatively low molecular weight region (*i.e.* between 14 and 18 kDa).

Shannon and Curson(1983) and Baas *et al.* (1983) reported that components in the diffusate obtained from the dialysis of seminal plasma against water under neutral conditions had a beneficial effect on sperm. Baas *et al.* (1983) reported that the motility of sperm deactivated by washing in Ficoll solution was sustained for much longer when incubated with the ultrafiltrate of this diffusate (*i.e.* material that passed through an ultrafilter of 500 Da molecular weight cut-off) while it was shortened when incubated with whole seminal plasma. On testing the various fractions from seminal plasma, Baas (1985) reported that the retentate of seminal plasma dialysed against water (molecular weight >10 kDa) was detrimental to sperm motility (*i.e.* the duration of motility was shortened). In the present study it was demonstrated that detrimental activity was present in both diffusate and the retentate of seminal plasma. Further fractionation of the diffusate (from a 12 kDa cut-off membrane) resulted in a fraction of molecular weight equal or greater than 5 kDa (*i.e.* the void volume of Sephadex G25 (designated as HMWwd in Fig. 10) which was more detrimental to sperm than the whole diffusate (Table 5B).

The low molecular weight component of the diffusate (LMW_{wd}) did not show any detrimental effects on the motility of sperm. It cannot be said that this fraction does not possess any components with a detrimental effect on sperm motility, as the effect may be masked due to the domination of the beneficial factor reported to be present by Baas *et al.* (1983). This presumed beneficial factor did not reverse the adverse effect of HMW_{wd} since the whole diffusate showed an adverse effect on sperm motility, however, it was less than the HMW_{wd} probably due to the presence of the presumed beneficial factor. Nevertheless, the agglutination effect of HMW_{wd} was reversed when it was in conjunction with the other factors in the whole diffusate. HMW_{wd} is the equivalent of the fraction in the ultrafilter retentate in the study of Baas (1985). Whether this fraction obtained by Baas (1985), of molecular weight less than 10 kDa and above 500 Da has any detrimental action on sperm is not known.

Seminalplasmin, a potently anti-sperm and antibacterial cationic protein present in seminal plasma (Shivaji *et al.*, 1984, Scheit *et al.*, 1985), has been reported by Reddy and Bhargava (1979) to have an estimated molecular weight between 10.6 kDa and 17 kDa. Later, Theil and Scheit (1983) isolated pure seminalplasmin with a molecular weight of 6.3 kDa. Shivaji (1988) in his review describes seminalplasmin as a 5.4 kDa, highly basic protein (isoelectric point 9.6) present in the seminal fluid at a concentration of approximately 500 µg/ml. Dialysing with membrane of 12 kDa cut-off will lead to excessive losses of seminalplasmin unless a special benzoylated tube is used (Theil and Scheit, 1983). The diffusate of seminal plasma dialysed against water

under neutral conditions in the present study would contain this component. The diffusate was examined for anti-sperm activity and was found to be active. The reduced antibacterial activity in the retentate of dialysed seminal plasma compared with whole seminal plasma may be due to the diffusion of seminalplasmin.

Hameed (1987) and Shannon *et al.* (1987) demonstrated that dialysis of seminal plasma, (pre-dialysed against water) in the presence of citrate (a poly-anionic agent) at pH 7.0 or alternatively under acidic conditions (pH 3.0) using a 3.5 kDa cut-off membrane, caused the antibacterial activity to diffuse slowly across the membrane, and the diffusion of the activity was continuous over several changes. Metal ion bridging may be involved in the formation of the aggregates that contribute to the antibacterial activity, since the antibacterial activity was found to diffuse across the 3.5 kDa cut-off membrane at neutral pH in the presence of 0.1 mol/l EDTA (P.C Molan, personal communication). This indicates that competing ionic forces would cause disaggregation of those aggregates that do not diffuse across the 12 kDa cut-off membrane in water at pH 7.0, but do diffuse across the membrane in the presence of citrate or when acidified (at pH 3 the negative charges on the carboxylic groups on the peptides will be suppressed).

In the present study (Sections 3.3 and 3.4) components with both anti-sperm and antibacterial activity had crossed the 3.5 kDa cut-off membrane and appeared in the diffusate when seminal plasma was dialysed against water at pH 3 or in the presence of citrate at pH 6.9 , indicating that the activity, at least in part, was due to small components of apparent molecular weight of less than 3.5 kDa. These components after diffusion reaggregate to a higher molecular weight confirming that disaggregation had occurred in the presence of acid or citrate.

These low molecular weight components that appear in the diffusate exert deleterious effects on sperm motility far more than the high molecular weight components. This supports the observation of Shannon and Curson (1983) and Garcia and Graham (1987) that the diffusate contains low molecular weight components which are detrimental to sperm and dialysis is beneficial for sperm survival and recovery of post-thaw motility.

Dialysis in citrate buffer removes the low molecular weight components from seminal plasma. This effect of dialysis is also influenced by the effects of citrate and dilution on the protein aggregates in seminal plasma in creating a new equilibrium between the high and the low molecular weight components. The period of dialysis is also

important for the disaggregating process. A complete diffusion of the low molecular weight components was not achieved even though dialysis was carried out for extended periods. The low molecular weight components in the diffusate need to be removed to establish a new equilibrium of damaging components in the retentate and the diffusate. Further fractionation of the retentate of seminal plasma dialysed against citrate buffer produced low molecular weight components (section 3.3.3).

The continuous disaggregation of seminal plasma proteins in the presence of citrate and the production of low molecular weight components has been reported by Collingwood (1990). An attempt was made to remove an inhibitory factor for amino acid oxidase from seminal plasma by dialysis against citrate buffer. This factor was reported to be of low molecular weight of <1 kDa. Although dialysis for a long period and several changes of the diffusate had substantially reduced this inhibitory factor, the retentate retained some inhibitory activity. It seems that this factor was released from the aggregates in seminal plasma and the amount removed by dialysis was replenished by continuous disaggregation (Collingwood, 1990).

The citrate diffusate appears to be a complex of low molecular weight components ranging between 2 and 16 kDa, as shown by SDS-PAGE under reducing conditions. These components reaggregate under native conditions to form proteins of much higher molecular weight (20-150 kDa). The process of disaggregation and a shift towards the low molecular weight area has recently been reported by Kameda *et al.* (1992) who chromatographed human seminal plasma under dissociating conditions (*i.e.* in the presence of 0.1% SDS). Kameda *et al.* (1992) reported that immunological activity seen in a peak of molecular weight of 669 kDa (under neutral conditions) appeared in the low molecular weight fraction of 15 to 20 kDa under dissociating conditions. The elution profile of seminal plasma was quite different under dissociation condition (*i.e.* in the presence of SDS): the peak at 669 kDa shrunk with corresponding increase in the peak at the 15 to 25 kDa region.

It is interesting that molecular size dictates the relative effects of different fractions on sperm motility. When the components are in the form of a macromolecular aggregate (*e.g.* HMWcitr and MMWcitr fractions), the effect on sperm motility is less than when they are in the dissociated form. Further fractionation (Section 3.3.3.) of these high molecular weight components produced fractions below 5 kDa which were more detrimental to sperm motility than the parent molecules. Similar observations have been made by Shannon *et al.* (1974, 1975) who described the transition of seminal plasma from one aggregated stage to another with enhanced antibacterial and anti-

sperm activity. This suggests that the anti-sperm activity might be present in bovine seminal plasma non-covalently bound to other molecules or aggregated with itself as a large molecule. It is likely that the aggregates of high molecular weight work as a macromolecular reservoir for the low molecular weight toxic molecules.

The disaggregated material comprised a small part of the aggregate and was in lesser concentration compared with the parent molecule, yet its action was greater. It appears that disaggregation not only releases the low molecular weight components but also augments their anti-sperm activity. A similar effect has been observed with a motility inhibitor from boar seminal plasma (Strzezek *et al.*, 1992). When the inhibitor was incubated along with seminal plasma for 30 min prior to addition to sperm, its effect was reduced. Similarly De Lamirande *et al.* (1984) observed that inhibition of motility by a low molecular weight motility inhibitor from bull seminal plasma was higher when this factor was in the free form (*i.e.* not associated with the macromolecule).

Fractions in the citrate diffusate (Section 3.3.2.2) have a concentration-dependent effect on sperm (Table 7). The detrimental effects on sperm motility were increased with the increasing concentration of the fraction (Tables 10 to 13). Moustafa and Meszarose (1980) and Pangawkar *et al.* (1988) reported increasing damage to sperm with an increasing seminal plasma concentration.

A curious aspect is the appearance of a large void volume peak upon chromatography of MMWcitr on Sephadex G15 at pH 1.7 (Section 3.3.3.), this having high anti-sperm activity but originating from the least active fraction (MMWcitr in Table 8) from the retentate. Compared with similar fractions obtained from the diffusate and the HMWcitr, its detrimental effect on sperm was much higher. It could be argued that this is due to the increased protein concentration of this fraction compared with that from the diffusate, although this is not the case with the HMWcitr fraction. It was presumed that this G15Vo fraction remained in an aggregated state along with the high molecular weight components but upon extended dialysis they disaggregate with time to release the sperm-damaging factor. Shannon and Curson (1983) reported that dialysis against 12 volume of citrate buffer for a period longer than 18 h decreased the post-thaw survival of sperm. The extended period of dialysis would result in a new equilibrium being established particularly of the G15Vo component. The presence of citrate in the diluent and the dilution of seminal plasma could further exacerbate the process of disaggregation leading to the release of the toxic components. Dialysis of seminal plasma against citrate buffer resulted in the appearance of low molecular

weight components in the diffusate in the present study. Compared with the dialysis against water it can be seen that the diffusate resulting from the dialysis against citrate contains more proteins or peptides.

When the fractions from the chromatography of the retentate of seminal plasma dialysed against citrate buffer (Fig. 16) were dialysed with a 3.5 kDa cut-off membrane an increase in the effect of the dialysed fraction over which they originated from was observed. This may be due to some components diffusing out of the dialysis membrane which greatly contribute to the anti-sperm activity either by attenuating or increasing the activity. A dose-dependent effect was demonstrated with all fractions, and a high activity was in the fraction obtained from the dialysis of the MMWcitr (Table 12) which originally showed the lowest anti-sperm activity (see Table 8).

Dialysis at acidic pH demonstrated that when ionic strength reduced, disaggregation occurs (Sections 3.3.3 and 3.4). The effect was similar to that seen with citrate dialysis. At pH 3, even after continuous removal of the diffusate, small molecular weight components were still detected by electrophoresis and gel permeation chromatography. A similar observation has been recorded by Shannon *et al.* (1987). Antibacterial activity from bovine seminal plasma diffused out of the dialysis membrane even after several changes of the diffusate at pH 3. Chromatography of boar seminal plasma at pH 4 on Sephadex G200 eluted most of the protein in the low molecular weight region (Davies *et al.*, 1975). This supports the hypothesis that at low pH, protein in seminal plasma disaggregates to lower molecular weights.

Dialysis against water at pH 3 caused some of anti-sperm activity to diffuse out over several changes. De Lamirande *et al.* (1984) also observed that the seminal plasma motility inhibition activity was reduced after dialysis under acidic conditions. However, they suggested that the diffused low molecular weight component had no intrinsic activity and its action is just an activator for the macromolecule which act as a sperm motility inhibitor. In the present study, anti-sperm activity diffused out the dialysis membrane with a 3.5 kDa molecular weight cut-off under acidic conditions. The diffused components showed detrimental effects on sperm without combining with the retentate. The retentate itself retained a high anti-sperm activity. It is clear that the diffused component(s) acted independently after it had dissociated from the macromolecule. The anti-sperm activity appeared in the diffusates over several changes and the extent of the activity was similar in the second, third and fourth diffusate (Table 14). The overall activity was increased markedly both in the diffusate

and the retentate after acid dialysis. Although the proteins in seminal plasma undergo disaggregation under acidic conditions, the components in the diffusate with apparent molecular weight less than 3.5 kDa and those in the retentate both reaggregate to a higher molecular weight under acidic and neutral pH, however, it is more pronounced in the latter.

It was argued by De Lamirande *et al.* (1984) that the low molecular weight fraction works as an activator for the high molecular weight fraction which retained a low level of inhibitory action. There is no description of the molecular weight of the macromolecule nor the effect of the dialysate itself. The present study has demonstrated that anti-sperm activity is spread over a wide range of molecular weights being more prominent in the low molecular weight fraction and the retentate of seminal plasma dialysed at pH 3.

A motility inhibitor from boar seminal plasma has been reported by Strzezek *et al.* (1992). It is a peptide of 5.7 kDa molecular weight obtained by fractionation with acetone and appeared in the diffusate of dialysis of this fraction. However, upon chromatography on Sephadex G200, the motility inhibition activity was localised in the fraction of high molecular weight (*i.e.* the first fraction eluted from the column with estimated molecular weight of 300 kDa or over). This shift in molecular weight may be due to an aggregation of the low molecular weight fraction to a higher molecular weight either with itself or in combination with other components in seminal plasma. It is also interesting that when the inhibitory fraction was incubated with whole seminal plasma, the inhibitory action was lessened and the longer the incubation the less inhibitory to sperm motility it became. In the present study, the higher molecular weight fractions were less detrimental to sperm motility than the low molecular weight ones. Further fractionation of the high molecular weight fraction under dissociating conditions generated components of low molecular weight that were more detrimental to sperm motility although they originated from the less active high molecular weight fraction. However, the low molecular weight fractions obtained after disaggregation varied in their activity and some were not detrimental to sperm motility.

Upon dialysis with acid, the diffusion of the low molecular weight components was continuous as material appeared in each of the four successive batches of diffusate. In all the batches of diffusate, low molecular weight components were present which significantly reduced the motility of sperm. The hypothesis is that the low molecular weight components in their native state remain as a macromolecular aggregate which

may serve as a reservoir. When the environment surrounding the sperm gets acidic due to metabolic products, it causes the release of the low molecular weight component from the macromolecule and they exert their toxic effect on sperm after a period of incubation with seminal plasma.

The form of aggregation also seems to dictate the relative amount of activity. The disaggregated components when they reaggregate to a higher molecular weight seem more potent in their anti-sperm activity than in their original state (*i.e.* before disaggregation). The retentate of seminal plasma dialysed at pH 3, upon chromatography under neutral conditions appeared to have high molecular weight components similar to those seen in seminal plasma. However, the major portion of the fraction was of low molecular weight. Fractions from seminal plasma under acidic conditions or seminal plasma subjected to pH reduction without fractionation retained activity higher than seminal plasma without acid treatment (Table 14). This higher activity may be explained by conformational changes in the shape and the size of the aggregates which may tend to have more active sites on the rearranged aggregates.

Disaggregation of high molecular weight aggregates under acidic conditions (pH 3.0) at 4°C, occurred and an equilibrium existed between the large anti-sperm aggregates and the small ones (Section 3.4.1). Although acidic conditions may affect the ionic interactions, thus dissociating the components that are held by these forces, other forces such as hydrophobic attraction may cause clustering which is probably responsible for the precipitate at pH 3. The solubility of the precipitated material was low in buffer and required without the addition of dimethyl sulphoxide indicating hydrophobicity. However, examination of the precipitated material revealed that it contained more that one component in this cluster although there was primarily a 14-16 kDa component. Complete separation of the anti-sperm activity was not achieved using acid as a disaggregating agent. Although partial disaggregation to the lower molecular weight was apparent, these fractions varied in their anti-sperm activity. This variation in activity within components having similar apparent molecular weights may be due to variation in the assembly of the molecule, being rich in the active components in the highly active fraction while the fraction with a low level of activity was poor in it.

Anion exchange chromatography (Section 3.4.4.3, Fig. 33) did not completely disaggregate the components in the DMSO soluble material, and it seemed that disruption of ionic interaction alone is not sufficient to completely disaggregate the molecule. The presence of a complex of low molecular weight components was

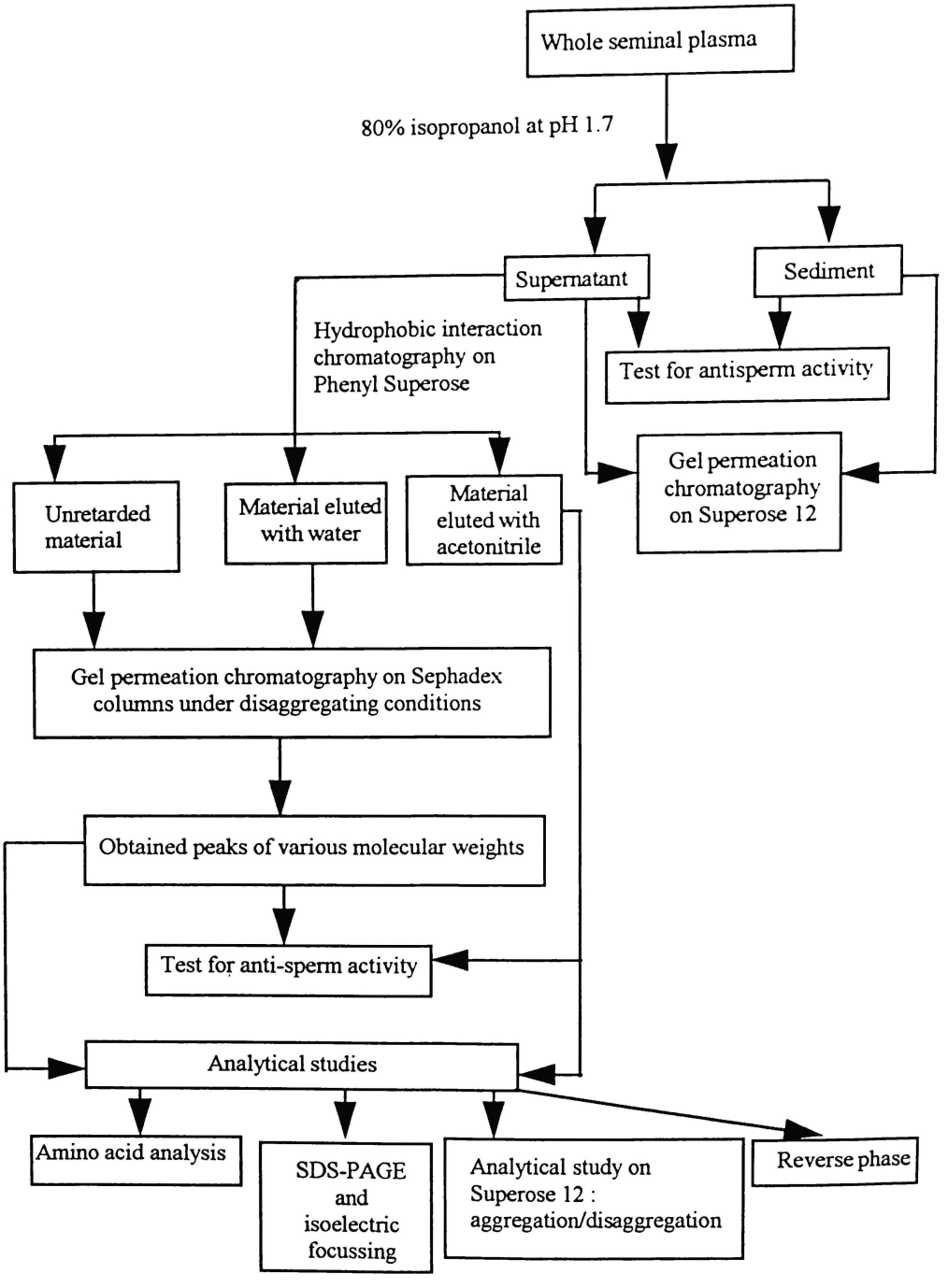
further shown by SDS-PAGE. It is clear that treatment with acid had caused disruption of the aggregate favouring the release of the relatively low molecular weight aggregates which are more detrimental to sperm than the macromolecule is.

Shannon *et al.* (1987) compared the acid and citrate diffusate, by cathodal electrophoresis at pH 9.2, and found similar bands in both of them. However, the bands were not clearly separated and the intensity was different. In the present study the acid diffusate and the citrate diffusate showed common bands upon SDS-PAGE on 20% gel under reducing conditions. It appears that each of the bands obtained by Shannon *et al.* (1987) was a form of an aggregate, since each band could be transformed into other several bands when subjected to electrophoresis.

The attempts to separate the anti-sperm component using citrate or acid as a disaggregating agent was only partially successful. Anti-sperm activity was associated with the low molecular weight component in the region below 5 kDa and the larger components >20 kDa. Dissociation in the presence of citrate and acid was not strong enough to completely dissociate the anti-sperm activity since it is charge-dependent and other forces are reported to contribute to the aggregating of protein in bovine seminal plasma (Hameed, 1987). The involvement of hydrophobic attraction was demonstrated by Hameed (1987) and Collingwood (1990). Thus breaking down the ionic and the hydrophobic attraction may disaggregate the molecule and separate the anti-sperm activity.

It was therefore decided to try separating the hydrophobic from the hydrophilic components. If disaggregation produced more active components then this would explain the role of the aggregates in preventing severe damage to sperm. On the other hand, if the disaggregated material is of less activity or has no activity on sperm then this will explain that the damaging component acts as an aggregate and not the individual components which the aggregates are made up of.

Flow chart for Section 4.1



CHAPTER FOUR

FURTHER ATTEMPTS TO DISSOCIATE THE ANTI-SPERM PEPTIDE FROM THE MACROMOLECULE

The observations from Chapter 3 led to the conclusion that the macromolecular aggregates of 500 and 250 kDa can be disaggregated under acidic conditions, dilution and in the presence of citrate to smaller aggregates. These small aggregates have high anti-sperm activity and pass through a 3.5 kDa dialysis membrane.

Within the dissociated material from the macromolecule, a stable aggregate of 20 kDa was reported by Hameed (1987). The stable aggregate requires harsh conditions to break down into its constituent components. Therefore, it was decided to influence this by breaking down the ionic and hydrophobic interactions that are responsible for reaggregation. There is evidence that when ionic forces are suppressed, hydrophobic attraction still contributes to aggregate formation (Hameed, 1987).

It seems that the components of low molecular weight in seminal plasma which were released from the macromolecular weight components favour being in the form of stable aggregates of relatively low molecular weight. When disaggregation takes place and components with apparent molecular weights of about 1-1.5 kDa are released, they will separate and when the conditions favour aggregation they form a larger molecule. Although acid will suppress the ionic interactions, it is possible that hydrophobic attraction will aid the formation of an aggregate. Shannon *et al.* (1987) observed that components which pass through a membrane with a 2 kDa molecular weight cut-off, tend to form an aggregate upon chromatography, even under acidic conditions.

In Chapter 3, the anti-sperm activity was found to reside in the small aggregate. The main focus in this chapter was to isolate the active anti-sperm component from this aggregate.

4.1 PRELIMINARY SEPARATION OF THE HYDROPHOBIC AND HYDROPHILIC COMPONENTS

The aggregates in bovine seminal plasma were described as being composed of basic hydrophilic and acidic hydrophobic peptides (Hameed, 1987). Thus, separation of these materials might be achieved by using a simple method involving precipitation

with isopropanol under acidic conditions. This was an attempt to interfere with the ionic and hydrophobic interactions that hold the aggregate together.

4.1.1 ISOPROPANOL PRECIPITATION

The pH of 5 ml of the retentate of dialysed seminal plasma was adjusted to 1.7 with HCl to suppress the ionic interactions. A pH of 1.7 was chosen because at extreme pH, hydrolysis may take place. It was then mixed with 20 ml of isopropanol and stirred quickly: precipitation resulted. The sediment and the supernatant were separated by centrifugation at 20 000 *g* for 30 min and both fractions were kept for further investigation.

4.1.1.1 SCREENING FOR ANTI-SPERM ACTIVITY OF THE ISOPROPANOL SUPERNATANT AND SEDIMENT

The isopropanol sediment was taken up with 20 ml of Milli Q water and the pH adjusted to 7, then the volume was reduced to 5 ml by rotary evaporation. It was then desalted on Sephadex G10 eluted with water so that no residue of isopropanol was present. The desalted material was reduced to 2.5 ml by rotary evaporation. There was no insoluble material.

The pH of the isopropanol supernatant was adjusted to 7 and rotary evaporated to dryness. The residue was then dissolved in 2.5 ml of water. This solution appeared to be cloudy. The solution of the isopropanol supernatant (2.5 ml) was centrifuged at 20 000 *g* for 10 min and the clear supernatant that resulted was separated from the small amount of insoluble material. To the insoluble material from the supernatant a few drops of dimethyl sulphoxide (DMSO) were added, and it was then taken up with 2.5 ml of water.

A 1 ml sample of each of the solutions of the isopropanol supernatant, the insoluble material in the supernatant that dissolved in dimethyl sulphoxide and the isopropanol sediment, were mixed 1:1 with DS14G diluent to be tested for anti-sperm activity.

To test the anti-sperm activity of a combination of all the three fractions mentioned above (*i.e.* isopropanol supernatant, the insoluble material in the supernatant that dissolved in dimethyl sulphoxide and the isopropanol sediment), 0.5 ml of each was mixed together (total volume 1.5 ml). The volume of this mixture was reduced t0 0.5 ml by rotary evaporation and the concentrate (0.5 ml) was then mixed 1:1 with DS14G diluent to be tested for anti-sperm activity.

A 100 µl aliquot from each of the prepared samples mentioned above was added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 16.

Table 16. The effect on the motility of washed bull sperm of the material in the supernatant and the sediment which resulted from the precipitation of seminal plasma with isopropanol .
The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		83.3±1.7^{a}	73.3±1.7^{a}
Isopropanol supernatant	1.35	75.0±2.9^{b}	48.3±3.3^{cd}
Isopropanol sediment	1.97	78.3±1.7^{b}	53.3±3.3^{c}
DMSO-soluble precipitate from supernatant	0.26	78.3±2.7^{b}	45.0±2.9^{d}
Combination of supernatant, the DMSO-soluble precipitate from supernatant and sediment	3.58	80.0±2.9^{ab}	60.0±2.9^{b}
DSP*	3.63	83.3±1.7^{a}	65.0±2.9^{b}

**Retentate of dialysed seminal plasma against water at 10% concentration for comparison.*
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

These results indicate that both the supernatant and the sediment significantly reduced the percentage of motile sperm when compared with the control. The sediment which was dissolved by the addition of dimethyl sulphoxide exerted high anti-sperm activity considering the relative amount of protein in the fractions. A similar observation was seen when using the sedimented material in the previous sections. It was interesting that the high effect of the fractions on sperm motility was reduced after re-combination: the activity of the combination of fractions was comparable to seminal plasma dialysed against water.

4.1.1.2 IDENTIFICATION OF THE AGGREGATES OF DIFFERENT MOLECULAR WEIGHT IN THE ISOPROPANOL SUPERNATANT AND SEDIMENT FROM SEMINAL PLASMA

An attempt was made to determine the composition of the isopropanol supernatant and sediment from seminal plasma and whether the components were in an aggregated form. The aim was to find the molecular weight of the aggregates. Accordingly, gel permeation chromatography on a Superose 12 column under neutral conditions was conducted.

A 100 µl sample from the solution of the dried isopropanol supernatant from seminal plasma was mixed 1:1 with 0.2 mol/l Tris-HCl at pH 7 then chromatographed on a Superose 12 column and eluted with 0.1 mol/l of Tris-HCl buffer, pH 7. The results (in Fig. 36) show peaks at the void volume, however, the bulk of the protein was eluted at the region of relatively low molecular weight (less than 67 kDa).

The solution of DMSO-soluble precipitate from the supernatant was chromatographed under the same conditions on the same column. The elution profile was similar to that shown in Fig. 30 which was obtained from the sediment dissolved in dimethyl sulphoxide that was obtained from the retentate of acid dialysis of seminal plasma as described in Section 3.4.4.1. The elution profile shows that the material eluted near the bed volume indicating low molecular weight.

The solution of the isopropanol sediment from seminal plasma was subjected to chromatography on the Superose 12 column in the same way. The results are shown in Fig. 37. Again, a peak at the void volume was seen, with the bulk of the material eluting in the region of lower molecular weight, between 70 and 6 kDa. There seems to have been some disaggregation caused by isopropanol that favours the lower molecular weight components. The treatment with isopropanol at pH 1.7 caused partial disaggregation as most of the material eluted at the relatively low molecular weight region.

As shown in Figs. 36 and 37, most of the components in the supernatant and the sediment were still in an aggregated form and comprised of a wide range of molecular weight components, however, mostly with molecular weights less than 67 kDa. To determine if combining these components would promote reaggregation to the original state, a combination of all fractions was chromatographed under neutral conditions. A 100 µl sample from the solutions of each fraction was taken. These were mixed together and the mixture diluted 1:1 with 0.2 mol/l Tris-HCl at pH 7 and

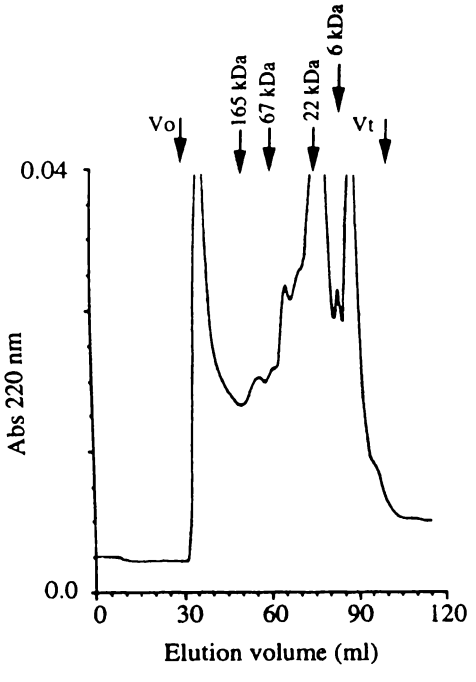


Fig. 36

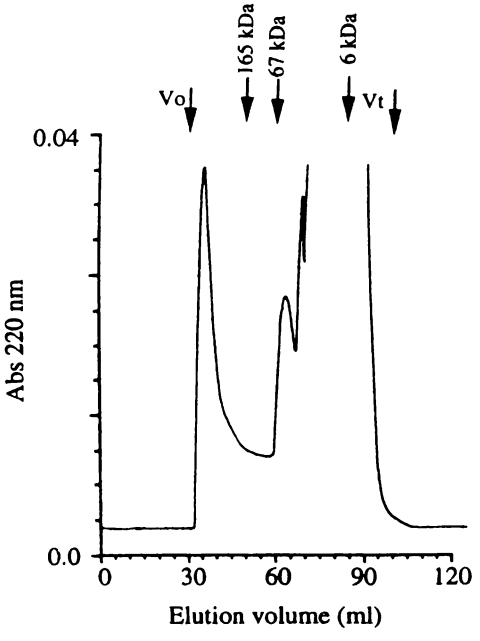


Fig. 37

Fig. 36: Gel permeation chromatography of the isopropanol supernatant from seminal plasma.
Column: preparative Superose 12.
Sample: 200 µl of the mixture of isopropanol supernatant 1:1 with 0.2 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 1 ml/min.

Fig. 37: Gel permeation chromatography of the isopropanol sediment.
Column: preparative Superose 12.
Sample: 200 µl of the mixture of isopropanol sediment 1:1 with 0.2 mol/l Tris-HCl at pH 7..
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 1 ml/min.

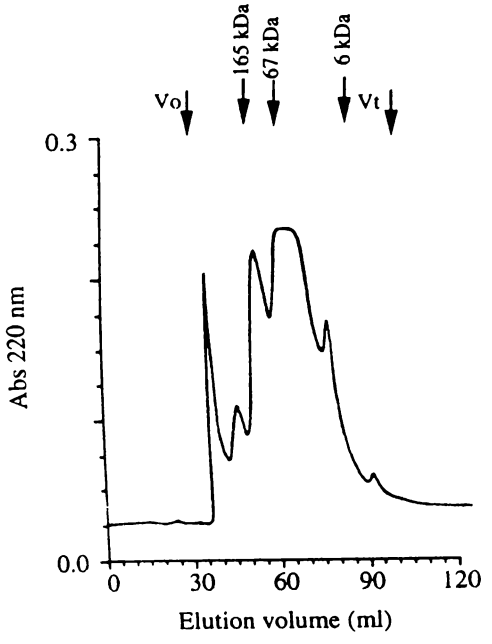


Fig. 38: Gel permeation chromatography of the combination of the supernatant, the sediments and the DMSO-soluble precipitate from the precipitation of seminal plasma with isopropanol.
Column: preparative Superose 12.
Sample: 400 µl of the mixture of the combination mixed 1:1 with 0.2 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 1 ml/min

incubated for 2 h at 37°C. A 400 µl sample of the mixture was chromatographed on the Superose 12 column under neutral conditions. The results (in Fig. 38) showed peaks with a wide range of molecular weight, with a shift towards the high molecular weight region (peaks of 67 and over 165 kDa) up to the void volume with estimated molecular weight of 300 kDa.

4.2 A STUDY OF THE ISOPROPANOL SUPERNATANT FROM SEMINAL PLASMA

4.2.1 CHROMATOGRAPHY ON PHENYL SUPEROSE COLUMN

An attempt to disaggregate the large molecular weight components using isopropanol in acidic conditions did not succeed in releasing the components in the aggregate. From the chromatography on Superose 12 column it was apparent that the material was still in its aggregated form, however, a major change in the profile of the supernatant and the sediment of isopropanol precipitation was seen when compared with the original profile of seminal plasma. A great shift toward the lower molecular weight was observed.

It was thought that the supernatant from the 80% isopropanol precipitation experiment would be richer in hydrophobic components than the sediment. Subjecting this supernatant to chromatography in which the hydrophobic components would bind to the column allowing the hydrophilic components to pass through would be a further way of separating these components. Thus, chromatography on a column of Phenyl Superose was carried out. Accordingly, 5 ml of the solution of the dried supernatant from the precipitation of seminal plasma with 80% isopropanol, prepared as described before (Section 4.1.1), was adjusted to pH 7 and rotary evaporated to dryness then dissolved in 5 ml of 1 mol/l of ammonium sulphate. The solution was centrifuged at 20 000 *g* for 10 min and from the clear supernatant a 1 ml sample was injected onto the Phenyl Superose column and eluted as detailed in Fig. 39. The chromatography was repeated 4 times to accumulate material.

The fractions comprising the unbound material that passed through the column with the ammonium sulphate (fractions from 2 to 10 min) from the five runs were pooled, diluted to 500 ml with Milli Q water and ultrafiltered through a membrane of 500 dalton molecular weight cut-off. The retentate (10 ml) was rotary evaporated to 2.5 ml. This material was further desalted on Sephadex G10 eluted with water and the material eluted at the void volume was reduced to 2.5 ml by rotary evaporation.

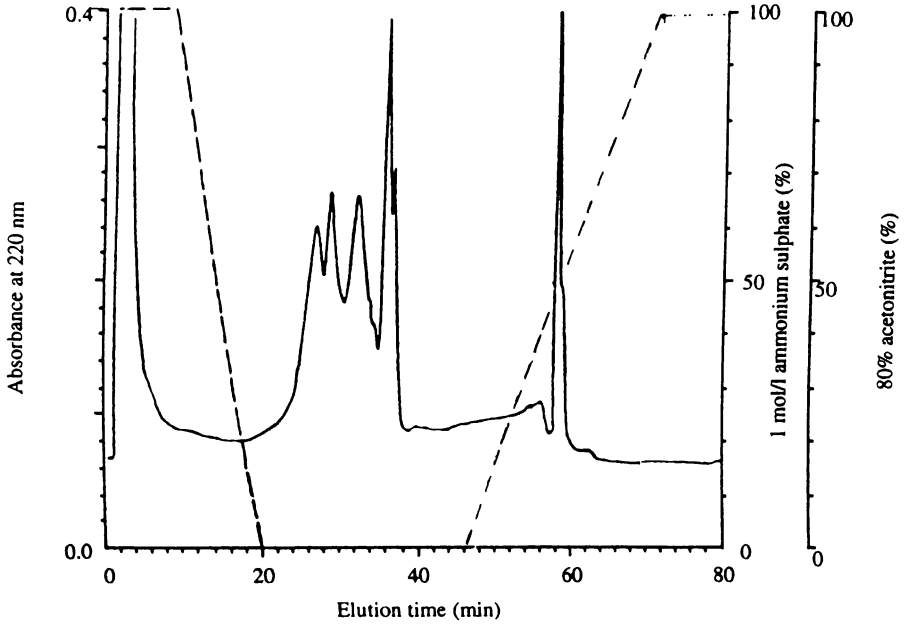


Fig. 39: Chromatography of the isopropanol supernatant from seminal plasma on Phenyl Superose.

Column: Phenyl Superose HR 5/5

Sample: 1 ml of isopropanol supernatant adjusted to 1 mol/l ammonium sulphate.

Eluents A: 1 mol/l ammonium sulphate

B: Milli Q water

C: 80% acetonitrile

Elution: 100% A for 10 min

0-100 % B in 10 min

100% B for 27 min

0-100% C in 23 min

Flow rate: 0.5 ml/min

The fractions containing material eluted from the Phenyl Superose with water were divided into two groups: the material that eluted early with water (from 24 to 36 min), and the material that eluted later with water (from 36 to 38 min). These pools of fractions were collected separately and each rotary evaporated to 2.5 ml. Finally, the material that was eluted with the acetonitrile gradient (from 54 to 60 min) was also collected, and rotary evaporated to dryness. To the residue a few drops of dimethyl sulphoxide was added and taken up with 2.5 ml water.

4.2.1.1 A STUDY OF THE PEAK ELUTED WITH AMMONIUM SULPHATE

Gel permeation chromatography on Superose 12 under neutral conditions

To determine whether the material eluted with the ammonium sulphate (from 2 to 10 min in Fig. 39) was in an aggregated form, it was chromatographed under neutral conditions on a Superose 12 column. A 100 µl sample from the concentrated desalted material of the peak which eluted with the ammonium sulphate (fractions 2 to 10 min in Fig. 39) was mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7 then chromatographed on the Superose 12 column eluted with 0.1 mol/l Tris-HCl buffer, pH 7. The results are shown in Fig. 40. As can be seen, several peaks eluted in the region of medium molecular weight (around 67 kDa) with a small peak in the high molecular weight region, however, the bulk of the protein eluted in the relatively low molecular weight region with estimated molecular weight less than 20 kDa. It appeared that the component was still in an aggregated form but failed to form the original aggregate as found in seminal plasma.

Gel permeation chromatography on Sephadex columns under acid conditions

To see if further chromatography on Sephadex under acidic conditions could cause further disaggregation, 2.4 ml of the concentrated desalted fractions eluted from 2 to 10 min in Fig. 39 was adjusted to pH 1.7, then held for 2 h at 4°C. It was then chromatographed in two lots on a column of Sephadex G25 as detailed in Fig. 41.

It can be seen that there was a peak eluted at the void volume area followed by other poorly resolved peaks. It was expected that the separation on Phenyl Superose and the chromatography on Sephadex under acidic conditions would cause further disaggregation of the component which was assumed to be hydrophilic. It seemed that the protein was still in an aggregated form. The estimated molecular weight for the void volume peak is equal to or greater than 5 kDa and the other peaks were less than 5 kDa.

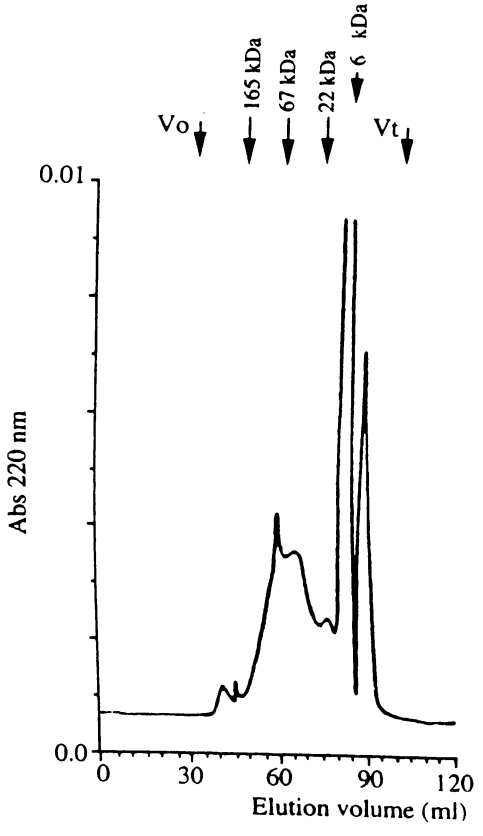


Fig. 40

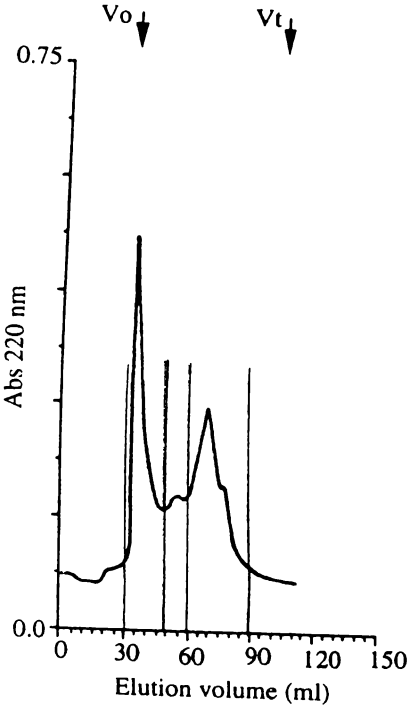


Fig. 41

Fig. 40: Gel permeation chromatography of the peak eluted with ammonium sulphate in Fig. 39 (fractions 2 to 10 min).
Column: preparative Superose 12.
Sample: 100 µl of the concentrate of fractions 2 to 10 min in Fig. 39 mixed 1:1 with 0.2 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 1 ml/min.

Fig. 41: Gel permeation chromatography of the peak eluted with ammonium sulphate in Fig. 39 (fractions 2 to 10 min).
Gel: Sephadex G25 SF.
Sample: 1.2 ml of the concentrated material adjusted to pH 1.7.
Eluent: 0.02 mol/l HCl, pH 1.7.
Flow rate: 1 ml/min.
Fraction volume: 5 ml.

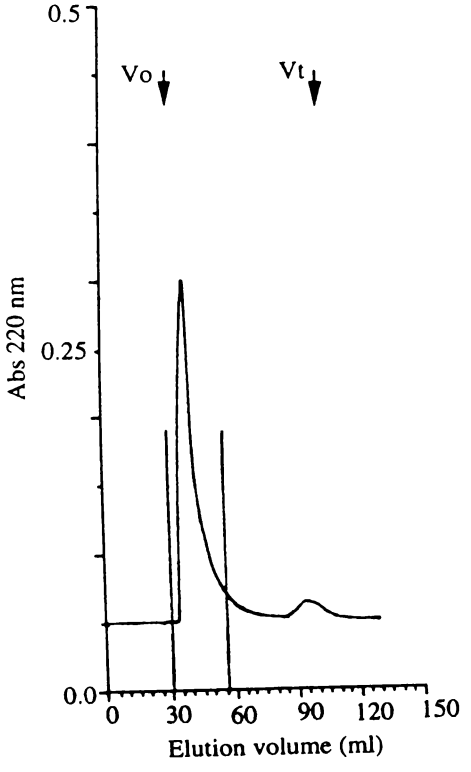


Fig. 42: Gel permeation chromatography of the peak eluted from 60 to 90 ml in Fig. 41.
Gel: Sephadex G15.
Sample: 2 ml of the concentrated material adjusted to pH 1.7.
Eluent: 0.02 mol/l HCl, pH 1.7.
Flow rate: 1 ml/min.
Fraction volume: 5 ml.

The fractions comprising the void volume peak in Fig. 41 (30 to 50 ml) from both runs were pooled, the pH was adjusted to 7 and the volume reduced to 2.4 ml (half of the original volume of the sample) by rotary evaporation. The fractions comprising the peak from 50 to 60 ml in Fig. 41 were collected, their pH was adjusted to 7 and concentrated to 2.4 ml (half of the original volume of the sample) by rotary evaporation. The fractions from 60 to 90 ml were collected and their volume was reduced to 2 ml, they were then chromatographed on a column of Sephadex G15 eluted with 0.02 mol/l HCl, pH 1.7 to remove any residual ammonium sulphate. The results are shown in Fig. 42. As can be seen the material eluted at the void volume indicating a molecular weight of 1.5 kDa or more. There was a small peak at the bed volume which was probably salt. The fractions comprising the void volume peak (fractions 30 to 55 ml) were pooled, the pH was adjusted to 7 and their volume was reduced to 2.4 ml by rotary evaporation.

Test for anti-sperm activity

A 1 ml sample from each of the processed fractions from chromatography on Sephadex G25 and Sephadex G15 was mixed 1:1 with DS14G diluent and retained for testing on sperm. To 1 ml of dialysed seminal plasma ammonium sulphate was added to reach a final concentration of 1 mol/l. It was then incubated for the same period as required for the chromatography on the Phenyl Superose column. After incubation, the material was desalted on Sephadex G10. This desalted seminal plasma was concentrated to 0.5 ml then mixed 1:1 with DS14G diluent to be tested for anti-sperm activity. A sample from the isopropanol supernatant that was obtained as described in Section 4.1.1 and prepared as in Section 4.1.1.1 was included in the test for anti-sperm activity. A 0.5 ml sample was mixed 1:1 with DS14G. Aliquots of 100 µl from the prepared samples were added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of $10\text{-}12 \times 10^6$ sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 17.

The hydrophilic components which were not retarded on the Phenyl Superose column reduced the percentage of motile sperm after 18 h incubation compared with the control, however, the components of higher molecular weight (fractions 30 to 50 ml and 50 to 60 ml from Fig. 41) did not show high anti-sperm activity and fraction 50 to 60 ml from Fig. 41 was significantly ($p>0.05$) less active than the retentate of dialysed seminal plasma treated with ammonium sulphate. The highest anti-sperm activity was found in the fraction of lowest molecular weight (void volume from

Sephadex G15 in Fig. 42). The reduction in the percentage of motile sperm was significantly greater than with the original seminal plasma and the activity was as great as that of the total activity of the supernatant from the isopropanol precipitation.

Table 17. Effect of the fractions in the peaks from Figs. 41 and 42 on the motility of washed bull sperm.
The values for motility are the percentage of motile sperm immediately and after 18 h incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		83.3±1.7^{a}	73.3±1.7^{a}
Fractions 35 to 50 ml in Fig. 41	0.21	81.7±1.7^{a}	66.7±1.7^{c}
Fractions 50 to 60 ml in Fig. 41	0.07	83.3±1.7^{a}	70.0±0.0^{b}
Vo peak of Fig. 42	0.17	80.0±2.9^{ab}	53.3±3.3^{d}
Isopropanol supernatant	1.35	75.0±2.9^{b}	48.3±3.3^{d}
DSP treated with ammonium sulphate*	3.63	83.3±1.7^{a}	65.0±2.9^{c}

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison (treated with ammonium sulphate like seminal plasma chromatographed)*
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The overall anti-sperm activity increased significantly when seminal plasma was fractionated with isopropanol and further chromatographed on Phenyl Superose. It seems likely that interference with the ionic and the hydrophobic interaction may have caused disaggregation releasing more active components of low molecular weight with high anti-sperm activity.

Gel permeation chromatography under disaggregating conditions on Superose 12

The components comprising the peak eluted with ammonium sulphate (fractions 2 to 10 min) in Fig. 39 were subjected to chromatography on the analytical Superose 12 column under dissociating conditions to gain a better idea of the material comprising the peak.

The result of the chromatography on Superose 12 under neutral conditions (Fig. 40) showed that the material comprising the peak which eluted with ammonium sulphate (fractions 2 to 10 min as in Fig. 39) was in an aggregated form of relatively high

molecular weight. An attempt was made to disaggregate the aggregate(s) of high molecular weight in this peak.

The chromatography on Phenyl Superose was repeated following the same procedures as in Fig. 39. The fractions comprising the ammonium sulphate peak (2 to 10 min) were collected and desalted on Sephadex G10. The desalted material was then rotary evaporated to dryness. The residue was then redissolved in 0.5 ml of 30% acetonitrile in 0.02 mol/l HCl, at pH 1.7, and held for 2 h at 4°C. A 250 µl aliquot of this solution was chromatographed on the analytical Superose 12 column eluted with 30% acetonitrile in 0.02 mol/l HCl at pH 1.7. The result is shown in Fig. 43a. As can be seen, the peaks of high molecular weight disaggregated to form a peak near the bed volume and a small peak at the bed volume. To determine whether dilution with acetonitrile under acidic conditions would cause further disaggregation, a 100 µl sample was diluted 10 times with 30% acetonitrile in 0.02 mol/l HCl, held for 2 h at 4°C then chromatographed on the same column. The result is shown in Fig. 43b. As can be seen, the material eluted in two peaks just before the bed volume. The peak before the bed volume got smaller while the peak at the bed volume got bigger. Further dilution (10 times) with 30% acetonitrile in 0.02 mol/l HCl resulted in disaggregation to a single peak at the bed volume with an estimated molecular weight of 1 kDa (see Fig. 43c).

A study of the re-aggregation of the components in Fig. 43

An attempt was made to study the re-aggregation capacity of the components obtained from the disaggregation (Fig. 43) of the peak which eluted with ammonium sulphate from the Phenyl Superose column. This was to determine whether the components resulting from this disaggregation have the capacity to reaggregate or if the dilution with acetonitrile under acidic conditions had affected this capacity.

To determine whether the two peaks which eluted near the bed volume in Fig. 43b contained the components necessary for aggregation it was decided to pool the two peaks together (fractions 18 to 20 ml). The chromatography of Fig. 43b was repeated 5 times and the two peaks were pooled (fractions 18 to 20 ml) and rotary evaporated to dryness. The residue was redissolved in 100 µl of 0.1 mol/l Tris buffer (pH 7), incubated for 2 h at 37°C and chromatographed with the same buffer on a Superose 12 column. The results indicated that an aggregation had taken place and peaks at the higher molecular weight region occurred up to 67 kDa as in Fig. 40: a shift toward the high molecular weight region occurred. The sample aggregated but not to the same extent as the original aggregate (Fig. 1 and 2), because the prominent

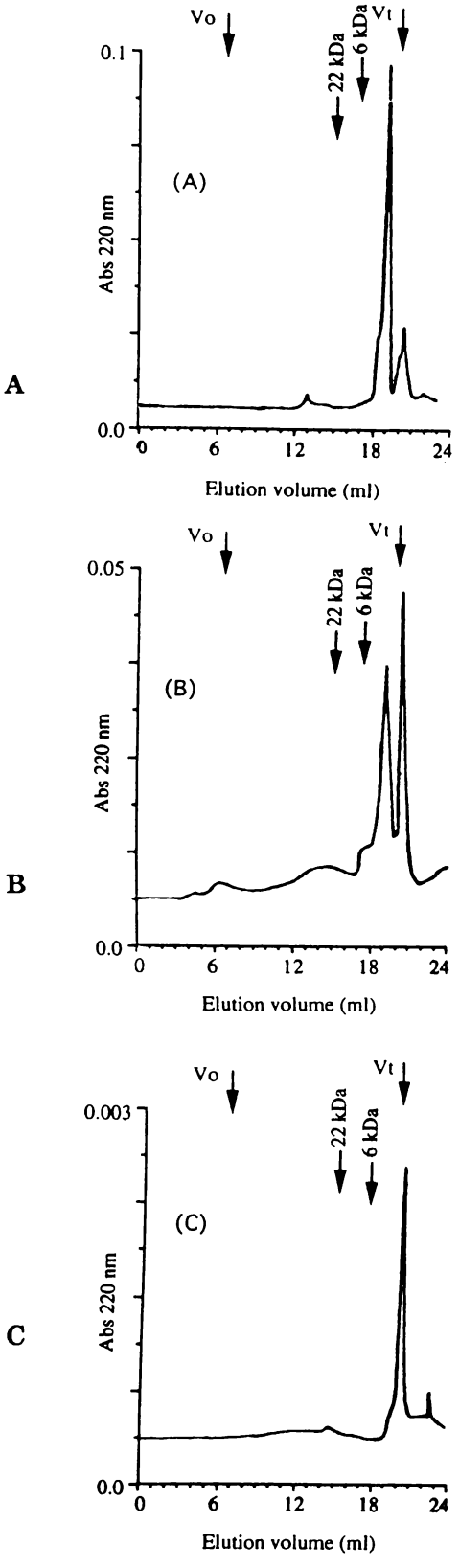


Fig. 43: Gel permeation chromatography on Superose 12 of the material eluted in the peak with ammonium sulphate of Fig. 39:
(A) without dilution (B) diluted 10X (C) diluted 100X
Column: analytical Superose 12 HR10/30
Samples: 250 µl of the redissolved residue in 30% acetonitrile in 0.02 mol/l HCl at pH 1.7
Eluent: 30% acetonitrile in 0.02 mol/l HCl
Flow rate: 0.5 ml/min

peak was at the region near the bed volume with estimated molecular weight less than 20 kDa.

Reaggregation study of the void volume peak from Fig. 42

Chromatography, under disaggregating conditions, of the fractions in the ammonium sulphate peak (Fig. 42) produced components of low molecular weight. Since this fraction of lowest molecular weight had the highest anti-sperm activity when compared with the other fractions from the ammonium sulphate peak, it was interesting to determine whether this peak could form aggregates of higher molecular weight. A 100 µl sample from the concentrate of the fractions of the void volume peak in Fig. 42 was mixed 1:1 with 0.2 mol/l Tris-HCl buffer at pH 7 then chromatographed on the Superose 12 column and eluted with 0.1 mol/l of Tris-HCl buffer, pH 7. The results are shown in Fig. 44. As can be seen, several peaks eluted at the higher molecular weight area (>20 kDa) indicating aggregation. However, the bulk of the protein eluted in the relatively low molecular weight area. It appeared that the components can form an aggregate but failed to form the original aggregate of seminal plasma.

Reverse phase chromatography

The void volume peak in Fig. 42 obtained by acid disaggregation of the hydrophilic components with an apparent molecular weight between 1.5 and 3.5 kDa was very active on sperm. Chromatography on Superose 12 under neutral condition showed that the material in this peak was in the form of an aggregate. It was thought that subjecting the components in this peak to reverse phase chromatography may result in further separation.

Accordingly, 500 µl of the concentrated material of the void volume peak in Fig. 42 was rotary evaporated to dryness, redissolved in 500 µl of 0.1% trifluoroacetic acid and then the sample was chromatographed on the reverse phase column. Trifluoroacetic acid was included as an ion pairing agent to promote the interaction of these components with the column matrix. The trifluoroacetic acid anion binds with the positively charged regions on the peptides and proteins while its hydrophobic region promotes adsorption to the reverse phase gel. As can be seen in Fig. 45 one unretarded peak (3 to 4 min) and several retained peaks on the column were eluted with the gradient: the first peak from 14 to 15 min, second peak from 15 to 17 min and a third unresolved peak from 18 to 22 min. This indicated differences in the hydrophobicity and complexity of the components. The fractions comprising each

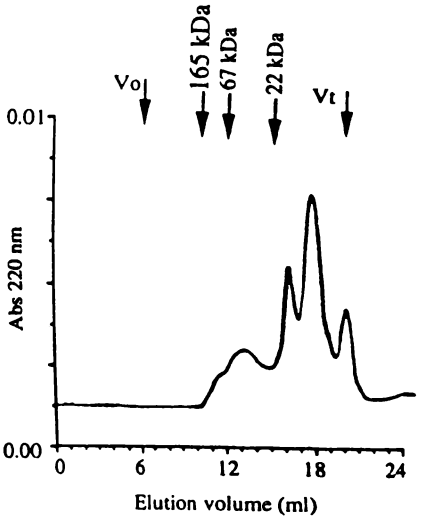


Fig. 44

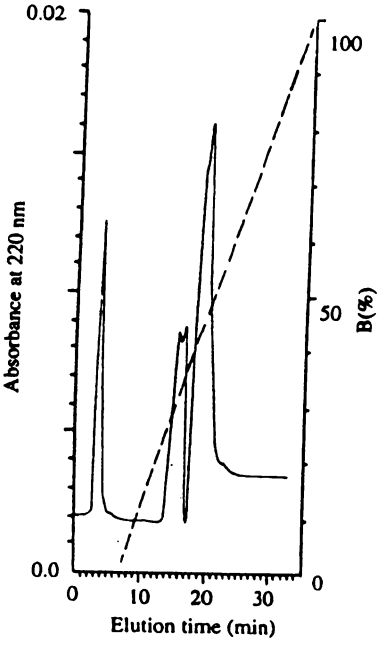


Fig. 45

Fig. 44: Gel permeation chromatography of the void volume peak in Fig. 42.
Column: analytical Superose 12 HR 10/30.
Sample: 200 µl of the mixture of the concentrate of the fractions in void volume peak in Fig. 42 with 0.2 mol/l Tris-HCl, at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, at pH 7.
Flow rate: 0.5 ml/min.

Fig. 45: Reverse phase chromatography of the material eluted in the void volume peak in Fig. 42.
Column: ProRPC HR 5/10
Sample: 500 µl of the material in 0.1% trifluoroacetic acid.
Eluent A: 0.1% trifluoroacetic acid.
Eluent B: 0.1% trifluoroacetic acid in 80% acetonitrile
Gradient: 0-100% B in 27 min
Flow rate: 1 ml/min.

peak in Fig. 45 were collected and freeze-dried. The residues were dissolved in 25 µl of 0.1 mol/l Tris-HCl, pH 7.

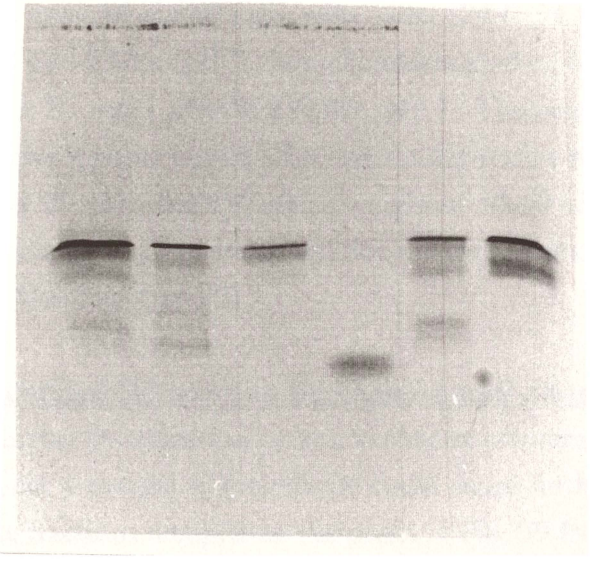
SDS-PAGE of the fraction in the peaks from Fig. 45

The composition of these peaks obtained from the reverse phase chromatography and their molecular weights was further investigated by SDS-PAGE. A 10 µl sample was mixed 1:1 with 5 times concentrated sample buffer and 1 µl of this mixture was loaded on a high density gel (20% polyacrylamide). The electrophoresis was performed on the PhastSystem and the gel was stained with silver stain. The results are shown in Fig. 46. All of the peaks contained more than one component. Peaks that eluted with the gradient showed several bands ranging between 4 and 12.5 kDa. A common band in the region of 12 kDa among all the fractions was observed. The peak that passed through the reverse phase column appeared to have similar bands in the region of 10 to 12.5 kDa as well as several faint bands in the higher molecular weight region.

Summary and conclusion:

The material that was not retarded by the Phenyl Superose column was a complex of molecules which retained the capacity for aggregation and anti-sperm activity. Further separation of these components, under dissociation conditions, produced low molecular weight fractions (1.5 kDa or less) with high anti-sperm activity and higher molecular weight fraction with low anti-sperm activity. The low molecular weight components reaggregated to higher molecular weight under neutral conditions. Dilution with acetonitrile under acidic conditions resulted in complete disaggregation to one peak at the bed volume of Superose 12 with an estimated molecular weight of 1 kDa.

Reverse phase chromatography did not seem to separate the small peptides in the highly active fraction (void volume of Fig. 42). Although several peaks eluted with the gradient in reverse phase chromatography, they were still in a complex form. This indicated that material which passed through the Phenyl Superose column unretarded was not separate components but was in the form of aggregates. These components were presumed to be hydrophilic. Although they separated on the reverse phase column into several peaks with varying degrees of hydrophobicity, SDS-PAGE showed that all fractions which eluted from the reverse phase column with the gradient were comprised of a complex of components with apparent molecular weights ranging between 3.5 and 12.5 kDa and had a common double band with estimated molecular weight between 10 and 12.5 kDa.



1 2 3 4 5 6

Fig. 46: SDS-PAGE under reducing conditions of the fractions in the peaks from Fig. 45 on a 20% polyacrylamide gel (high density PhastGel).
Lane 1: fractions in 18 to 22 min peak. Lane 2: fractions in 15 to 17 min peak. Lane 3: cytochrome c (12.5 kDa). Lane 4: insulin B chain (3.4 kDa) Lane 5: fractions in 14 to 15 min peak. Lane 6: fractions in non-retained peak (3 to 4 min).

4.2.1.2 A STUDY OF PEAKS WHICH ELUTED EARLY WITH WATER FROM THE CHROMATOGRAPHY ON THE PHENYL SUPEROSE COLUMN (FRACTIONS 24 TO 36 MIN IN FIG. 39)

The material which eluted with water from the column of Phenyl Superose is presumably hydrophobic since it bound to Phenyl Superose. This material was subjected to further investigation to determine the composition and wether disaggregation of the macromolecular weight aggregates had occurred.

Gel permeation chromatography on Superose 12 under neutral conditions

An attempt was made to further investigate the composition and the aggregation capacity of the material eluted early with water. A 100 µl sample from the concentrate of the material eluted early with water (24 to 36 min, Fig. 39) was mixed 1:1 with 0.2 mol/l Tris-HCl at pH 7, then chromatographed on the Superose 12 column eluted with 0.1 mol/l Tris-HCl buffer, pH 7. The results are shown in Fig. 47. As can be seen, several peaks eluted with various molecular weights. A major peak eluted in the region of estimated molecular weight of 50-60 kDa. It appeared that the components were in aggregated forms, but not similar to the original aggregates seen in seminal plasma (see Fig. 1 and 2).

Gel permeation chromatography on Sephadex under disaggregating conditions

To determine if further fractionation on the Sephadex columns under acidic conditions and in the presence of acetonitrile could cause further disaggregation, the concentrate of the fractions comprising the peaks in Fig. 39 from 24 to 36 min was further processed. To 2.4 ml of the concentrate was added acetonitrile (to 30%, v/v) and HCl to pH 1.7 and the final volume adjusted to 4 ml with the addition of 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. It was then held for 2 hours at 4°C and chromatographed on a Sephadex G25 column eluted with 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. Three peaks were obtained (shown in Fig. 48): one peak eluted at the void volume (30 to 45 ml) followed by another peak (45 to 75 ml) and a peak at the bed volume (75 to 110 ml).

It appeared that the proteins were still in an aggregated form as the estimated molecular weight for the void volume peak was equal to or over 5 kDa and the second peak between 1.5 and 5 kDa.

The fractions comprising the void volume peak in Fig. 48 (30 to 45 ml) were pooled, the pH adjusted to 7 and the volume reduced by rotary evaporation to 2.4 ml. The material was desalted on Sephadex G10 eluted with water and concentrated by rotary

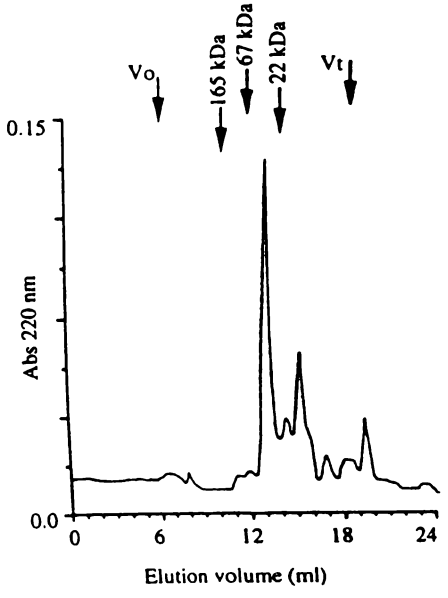


Fig. 47

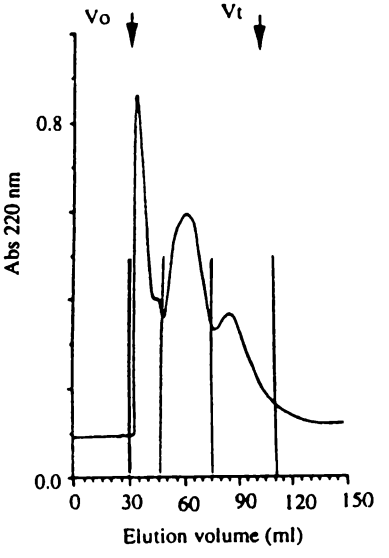


Fig. 48

Fig. 47: Gel permeation chromatography of fractions 24 to 36 min eluted with water in Fig. 39.
Column: analytical Superose 12 HR 10/30.
Sample: 100 µl of the concentrate of fractions 24 to 36 min in Fig. 39 mixed with 100 µl of 0.2 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 0.5 ml/min.

Fig. 48: Gel permeation chromatography of the peak eluted with water from 24 to 36 min in Fig. 39.
Gel: Sephadex G25 SF.
Sample: 4 ml of the concentrated material in 30% acetonitrile in 0.02 mol/l HCl, pH 1.7.
Eluent: 30% acetonitrile in 0.02 mol/l HCl, pH 1.7.
Flow rate: 1 ml/min
Fraction volume: 5 ml.

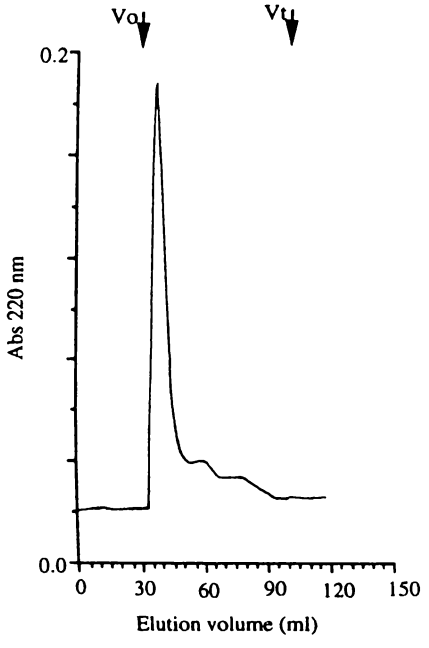


Fig. 49: Gel permeation chromatography of the fractions from 75 to 110 ml in Fig. 48.
Gel: Sephadex G15.
Sample: 2 ml of the concentrated material in 30% acetonitrile in 0.02 mol/l HCl, pH 1.7.
Eluent: 30% acetonitrile in 0.02 mol/l HCl, pH 1.7.
Flow rate: 1 ml/min.
Fraction volume: 5 ml.

evaporation to 2.4 ml again. The fractions comprising the peak from 45 to 75 ml in Fig. 48 were pooled, the pH adjusted to 7 and concentrated to 2.4 ml following the same procedure as above. The fractions from 70 to 110 ml were pooled and their volume was reduced to 2 ml and chromatographed on a Sephadex G15 column eluted with 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. One major peak at the void volume was obtained (Fig. 49), with some very minor peaks in the lower molecular weight region. The fractions comprising the major peak (fractions 35 to 45 ml in Fig. 49) were pooled, the pH was adjusted to 7, the volume reduced to 2.4 ml, and desalted on Sephadex G10 eluted with water then concentrated to 2.4 ml by rotary evaporation.

Test for anti-sperm activity

All the desalted fractions obtained from the chromatography on Sephadex G25 and Sephadex G15 were diluted 1:1 with DS14G and tested for anti-sperm activity. A sample of the retentate of seminal plasma that had been dialysed against water was also prepared. Aliquots of 100 µl from the prepared samples were added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are in Table 18.

Table 18. Effect of the fractions in the peaks from Figs. 48 and 49 on the motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		83.3±1.7^a	73.3±1.7^a
Fractions 30-45 ml in Fig. 48	0.19	81.7±1.7^{ab}	55.0±2.9^c
Fractions 45 to 75 ml in Fig. 48	0.25	83.3±1.7^a	40.0±2.9^d
Fractions 35 to 45 ml in Fig. 49	0.09	81.7±1.7^{ab}	73.3±1.7^a
Fractions 24 to 36 min in Fig. 39	0.56	78.3±1.7^b	58.3±3.3^c
DSP*	3.63	83.3±1.7^a	65.0±2.9^b

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison.*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

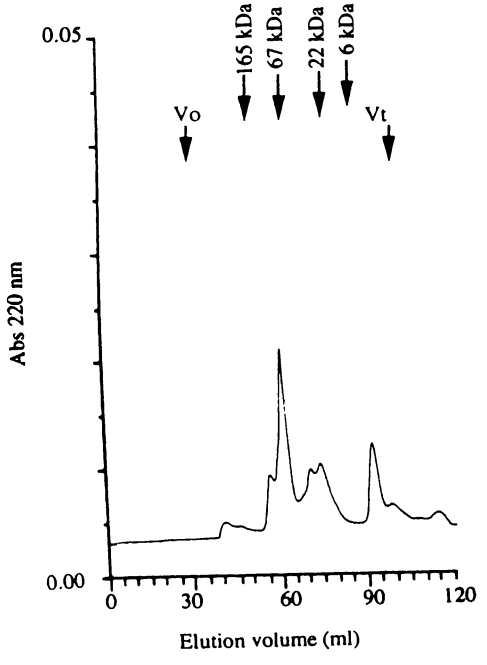
The material which eluted early with water from the Phenyl Superose column (fractions 24 to 36 min in Fig. 39) significantly reduced the percentage of motile sperm compared with the control after 18 h incubation. Fractions 30 to 45 ml from Fig. 48 (with estimated molecular weight of 5 kDa or greater) showed similar activity against sperm. Fractions 45 to 75 ml from Fig. 48 (with estimated molecular weight between 1.5 and 5 kDa) possessed the highest activity and it was significantly different from the other fractions. It is interesting that the whole fraction eluted early with water from Phenyl Superose, from which fraction 45 to 75 ml originated, did not reduce the percentage of motile sperm as much as fraction 45 to 75 ml. The components of lower molecular weight (fractions 35 to 45 ml from Fig. 49) did not reduce the percentage of motile sperm and there was no significant difference from the control. It appears that separation of the hydrophobic components on Sephadex columns in the presence of acetonitrile under acidic conditions had affected the activity in some fractions and released components which did not affect the motility of sperm. However, it also released a component(s) which significantly reduced the percentage of motile sperm.

Further investigation of the components in the fractions eluted early with water from the chromatography on Phenyl Superose as shown in Fig. 39

Gel permeation chromatography under neutral conditions of the components in the peaks from the chromatography shown in Fig. 48

The peak eluted at the void volume (30 to 45 ml in Fig. 48) was subjected to further study on the Superose 12 column under neutral conditions to determine if the components in this peak have the ability to form an aggregate of large molecular weight similar to the original ones in seminal plasma. A 100 µl sample from the concentrate of the fractions in the void volume peak (fractions 30 to 45 ml in Fig. 48) was mixed 1:1 with 0.2 mol/l Tris-HCl buffer at pH 7 then chromatographed on the Superose 12 column and eluted with 0.1 mol/l Tris-HCl buffer, pH 7. The results are shown in Fig. 50. From the profile it appeared that the material was composed of several components eluting in several peaks with a variety of molecular weights up to 100 kDa.

The fractions from 45 to 75 ml shown in Fig. 48 were also subjected to similar investigation on the Superose 12 column prepared and treated in the same manner as with the void volume peak. The results shown in Fig. 51 indicate that this material which had an apparent molecular weight of less than 5 kDa on Sephadex G25 (under



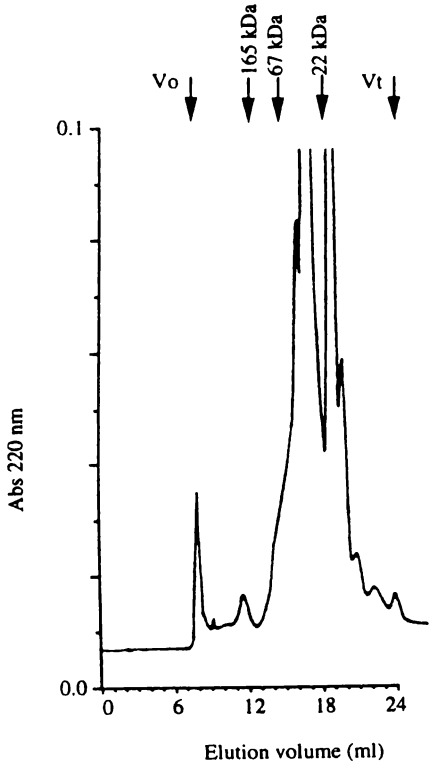


Fig. 50

Fig. 51

Fig. 50: Gel permeation chromatography of fractions in the void volume peak (30 to 45 ml) in Fig. 48.
Column: preparative Superose 12.
Sample: 200 µl of the mixture of the concentrate of fractions in the void volume peak in Fig. 48 mixed 1:1 with 0.2 mol/l Tris-HCl, at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 1 ml/min.

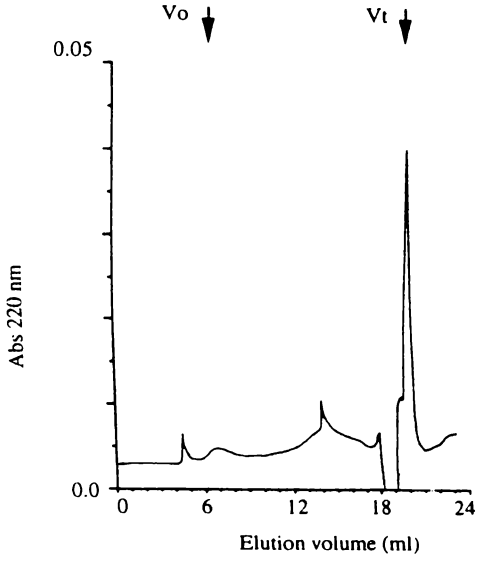
Fig. 51: Gel permeation chromatography of fractions 45 to 75 ml in Fig. 48.
Column: analytical Superose 12.
Sample: 200 µl of the mixture of the concentrate of fractions 45 to 75 ml in Fig. 48 mixed 1:1 with 0.2 mol/l Tris-HCl, at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 1 ml/min.

disaggregation conditions) had the ability to form aggregates with a molecular weight higher than 5 kDa. Several peaks, from the bed volume with estimated molecular weight from 1 kDa up to the void volume of Superose 12 (with estimated molecular weight of 300 kDa) were observed indicating aggregation, however, most of the material was less than 67 kDa.

The material in the peak that eluted in fractions 35 to 45 ml in Fig. 49 was also chromatographed under neutral conditions on the Superose 12 column. A 100 µl sample of the concentrate of this material was mixed 1:1 with 0.2 mol/l Tris-HCl buffer at pH 7 then chromatographed on the Superose 12 column eluted with 0.1 mol/l Tris-HCl buffer, pH 7. This time the material in this peak failed to reaggregate and eluted in one peak at the bed volume with an estimated molecular weight of 1 kDa (Fig. 52). Since the material failed to reaggregate at pH 7 it was decided to rechromatograph the material on the Sephadex G25 column to find its molecular weight. Accordingly, the Sephadex G25 column was equilibrated with 0.1 mol/l Tris-HCl, pH 7. A sample (250 µl) of the concentrate of the fractions from the peak (35 to 45 ml in Fig. 49) was mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7, incubated for 2 h at 37°C, then chromatographed on a Sephadex G25 column eluted with 0.1 mol/l Tris-HCl, pH 7. It was eluted at the bed volume with an estimated molecular weight of 1 to 1.5 kDa (Fig. 53). There was no peak in the higher molecular weight region. This result confirmed the results in Fig. 52 where the material failed to form a higher molecular weight aggregate and eluted in a single peak at the bed volume of Superose 12.

Disaggregation/reaggregation of the components in the peaks from Fig. 48
An attempt was made to disaggregate the high molecular weight aggregate(s) in the fractions comprising the peaks in Fig. 48.

Accordingly, 0.5 ml of the material which eluted from the column at the void volume (30 to 45 ml) in Fig. 48 was rotary evaporated to dryness. The residue was then redissolved in 0.5 ml of 30% acetonitrile in 0.02 mol/l HCl, at pH 1.7. A 100 µl sample of this solution was diluted 10 times with 30% acetonitrile in 0.02 mol/l HCl, at pH 1.7 then chromatographed on the Superose 12 column and eluted with the same solvent. The results in Fig. 54a showed a shift in elution of the components toward the bed volume of the column indicating disaggregation of the components. When the sample was further diluted (40 times) with 30% acetonitrile in 0.02 mol/l HCl at pH 1.7 and then held at 4°C for 2 h, disaggregation to a peak at the bed volume with an estimated molecular weight of 1 kDa and a minor retarded peak (see



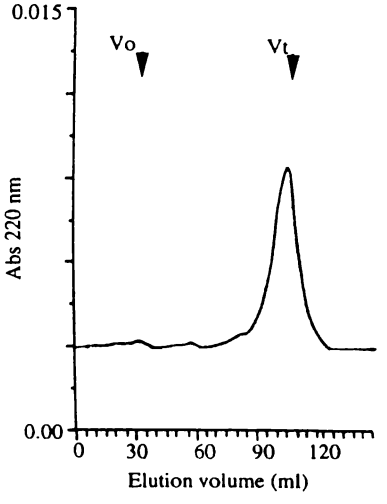


Fig. 52

Fig. 53

Fig. 52: Gel permeation chromatography of fractions 35 to 45 ml in Fig. 49.
Column: analytical Superose 12.
Sample: 200 µl of the mixture of the concentrate of fractions 30 to 55 ml in Fig. 49 with 0.2 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 0.5 ml/min.

Fig. 53: Gel permeation chromatography of fractions 35 to 45 ml in Fig. 49.
Column: Sephadex G25SF.
Sample: 0.5 ml of the mixture of the concentrate of fractions 35 to 45 ml in Fig. 49 with 0.2 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 1 ml/min.

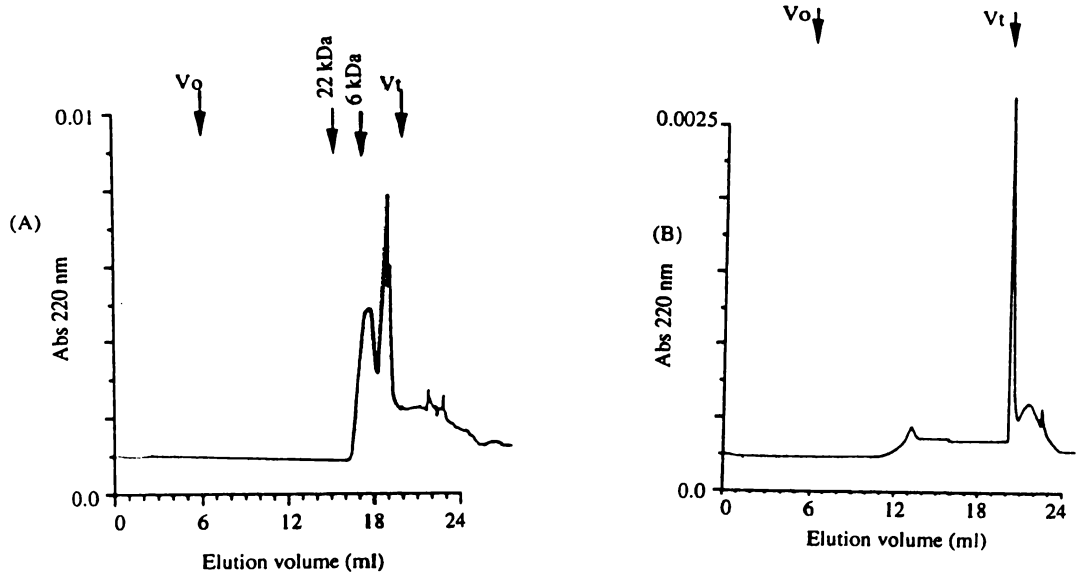


Fig. 54: Gel permeation chromatography of the material in the void volume peak in Fig. 48
(a) without dilution (b) diluted 40X
Column: analytical Superose 12 HR10/30
Samples: 100 µl of the redissolved residue of fraction 30 to 45 ml in Fig. 48 in 30% acetonitrile in 0.02 mol/l HCl at pH 1.7
Eluent: 30% acetonitrile in 0.02 mol/l HCl

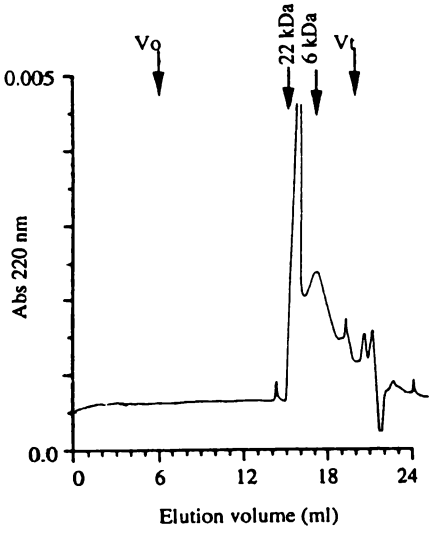


Fig. 55: Gel permeation chromatography of the components in the bed volume peak in Fig. 54b.
Column: analytical Superose 12.
Sample: 200 µl of the concentrate of fractions 19.5 to 22 ml in Fig. 54b at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 0.5 ml/min

Fig. 54b) took place. The disappearance of the other peaks may have occurred because further dilution had caused disaggregation to their components or because the components comprising the aggregate had been eluted together at the bed volume. This disaggregation study highlight the importance of the ionic and the hydrophobic interaction between the components and that dilution with acetonitrile in acid caused breakdown of these forces resulting in one peak at the bed volume.

Dilution may affect the concentration of each of the components reaching a stage where the stability of the aggregate was lost. Thus, the components would disaggregate.

Disaggregation to one peak at the bed volume meant that the components which were responsible for aggregation also eluted in this peak. If this is the case then the components in this peak must be capable of forming the aggregate if the disaggregating conditions are removed. An attempt to study the re-aggregation was made to determine whether the components resulting from this disaggregation were the same ones which the aggregates consisted of and whether the dilution had caused any disturbance to the capacity of these materials to form the original aggregates. Thus, repeated chromatography (20 times) as in Fig. 54b was performed to accumulate material. The pooled fractions (19.5 to 22 ml) from the repeated chromatography were freeze-dried and reconstituted in 200 µl of 0.1 mol/l Tris-HCl buffer at pH 7, incubated for 2 h at 37°C, then chromatographed on the Superose 12 column as detailed in Fig. 55. There was a shift in the elution profile and several peaks were obtained, indicating that the components in the bed volume peak had the ability to form an aggregate, however, they failed to form aggregates higher than 20 kDa. Disaggregation brought about by acetonitrile, acid, and dilution may be reversed if these conditions were removed. Not achieving full aggregation may be due to the aggregate formation component being present in too low a concentration.

The material in the second peak in Fig. 48 (fractions 45 to 75) was subjected to a similar disaggregation study. The result was similar to that shown in Fig. 54b. The material disaggregated to one peak at the bed volume with 100 times dilution. The disaggregated material when pooled, concentrated and rechromatographed under neutral conditions as above reaggregated to higher molecular weight, up to the area near the void volume.

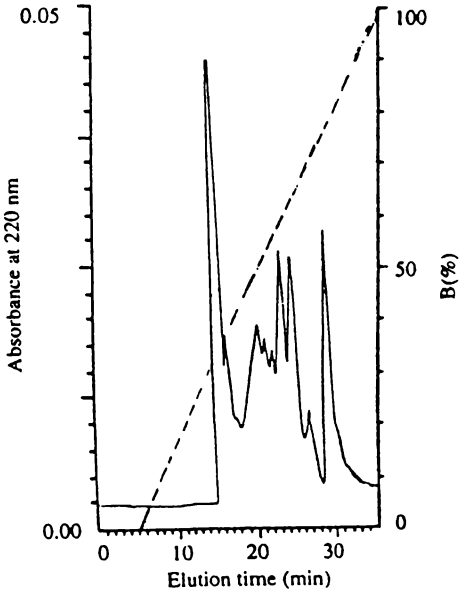
Reverse phase chromatography of the fractions from the chromatography shown in Figs. 48 and 49

Chromatography on Superose 12 showed that the material in fractions 30 to 45 ml and 45 to 75 ml in Fig. 48 still existed in the form of an aggregate. These components are hydrophobic in nature since they were retarded on the Phenyl Superose column. In further attempts to study the hydrophobic component, a reverse phase chromatography was performed on a ProRPC and PepRPC reverse phase columns to further investigate the nature of the material in the fractions from the chromatography shown in Figs. 48 and 49.

Accordingly, 0.25 ml of the material eluted in the void volume peak (fractions 30 to 45 ml in Fig. 48) was rotary evaporated to dryness. The dried material was redissolved in 0.25 ml of 5 mmol/l tetrabutylammonium sulphate. This was included as an ion pairing agent to promote the interaction of the components with the column matrix by binding ionically with the negatively charged regions on the peptides and proteins, while its hydrophobic region promotes adsorption to the reverse phase gel. The hydrophobic components of the aggregates were expected to be predominantly anionic (Collingwood, 1990). A 200 µl sample was chromatographed on the ProRPC reverse phase column as detailed in Fig. 56. From the results (Fig. 56) it can be seen that the material eluted in several peaks with the gradient. This separation indicated that the material was a complex of components with different degrees of hydrophobicity as some of them did not bind to the column.

Similarly, the material which eluted in the second peak (fractions 45 to 75 ml) in Fig. 48 was also subjected to reverse phase chromatography. A 0.25 ml sample of this material was rotary evaporated to dryness and the residue was redissolved in 0.25 ml of 5 mmol/l tetrabutylammonium sulphate, then 200 µl was chromatographed on the ProRPC HR 5/10 reverse phase column. The result in Fig. 57 shows again several peaks eluted with the gradient indicating that this material was a complex of components with different degrees of hydrophobicity.

The peak eluted in fractions 35 to 45 ml in Fig. 49 was subjected to similar chromatography on a PepRPC 5/5 reverse phase column which is recommended for the separation of peptides with molecular weights of less than 6 kDa. The results in Fig. 58 show that only one peak eluted with the gradient.



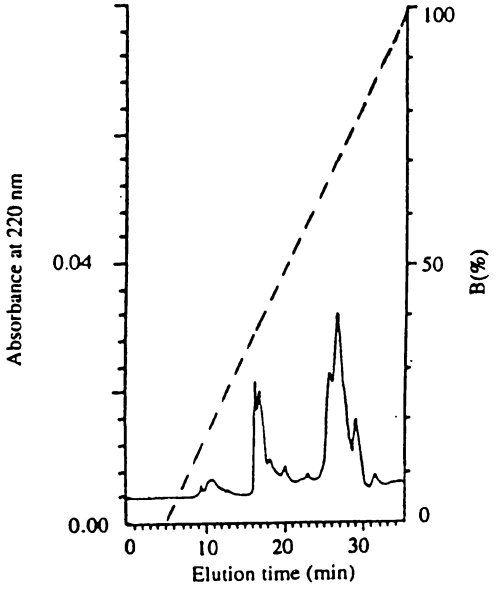


Fig. 56

Fig. 57

Fig. 56: Reverse phase chromatography of the material eluted in the void volume peak in Fig. 48
Column: ProRPC HR 5/10
Sample: 200 µl of the dried material from fractions 30 to 45 ml in Fig. 48 redissolved in 5 mmol/l tetrabutylammonium sulphate.
Eluent A: 5 mmol/l tetrabutylammonium sulphate
Eluent B: 80% acetonitrile in 5 mmol/l tetrabutylammonium sulphate.
Gradient: 0-100% B in 30 min
Flow rate: 1 ml/min.

Fig. 57: Reverse phase chromatography of the material eluted in the second peak in Fig. 48 (fractions 45-75 ml)
Column: ProRPC HR 5/10
Sample: 200 µl of the dried material from fractions 45 to 75 ml in Fig. 48 redissolved in 5 mmol/l tetrabutylammonium sulphate .
Eluent A: 5 mmol/l tetrabutylammonium sulphate
Eluent B: 80% acetonitrile in 5 mmol/l tetrabutylammonium sulphate.
Gradient: 0-100% B in 30 min
Flow rate: 1 ml/min.

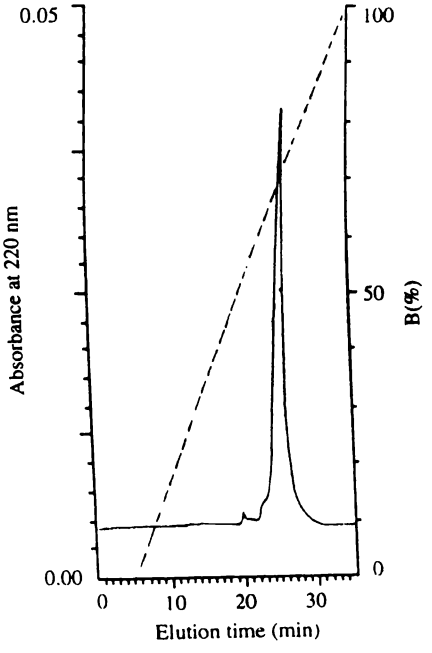


Fig. 58: Reverse phase chromatography of the material eluted in fractions 35-45 ml in Fig. 49.
Column: PepRPC HR 5/5
Sample: 200 µl of the dried material from fractions 35 to 45 ml in Fig. 49 redissolved in 5 mmol/l tetrabutylammonium sulphate .
Eluent A: 5 mmol/l tetrabutylammonium sulphate
Eluent B: 80% acetonitrile in 5 mmol/l tetrabutylammonium sulphate.
Gradient: 0-100% B in 30 min
Flow rate: 1 ml/min.

SDS-PAGE of the fractions in Figs. 48 and 49

Gel permeation and reverse phase chromatography demonstrated the complexity of the components in Fig. 48, however, fractions 35 to 45 ml in Fig. 49 appeared to be a single component. The composition and the molecular weights of the components which make up the peaks in Figs. 48 and 49 were further analysed by SDS-PAGE under reducing conditions. Accordingly, 10 µl of each of the concentrate of fractions comprising the peaks in Figs. 48 and 49 was mixed 1:1 with 5 times concentrated sample buffer. From the mixture, 1 µl was loaded on a high density (20%) polyacrylamide gel. The electrophoresis was performed on the PhastSystem. The results are in Fig. 59. Both the peaks in Fig. 48 contained more than one component, but the peak (fractions 35 to 45 ml) from Fig. 49 showed one band in the region with estimated molecular weight of 2 kDa. This result confirms the purity of the peptide from Fig. 49 and confirmed the estimated molecular weight from the chromatography on Superose 12 (Fig. 52) and Sephadex G25 (Fig. 53).

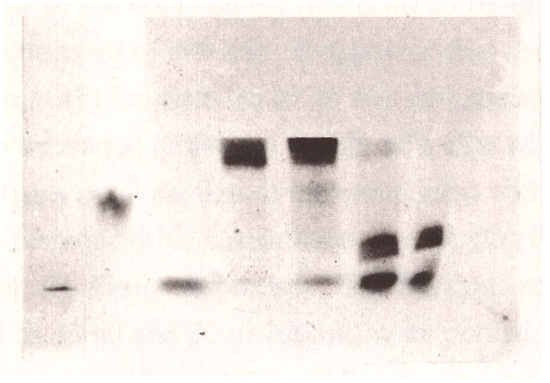
Isoelectric focussing determination

The fractions comprising the peak in Fig. 49 seemed to be a pure component as determined by gel permeation, reverse phase and SDS-PAGE. To determine the isoelectric point of the material eluted in the peak (fractions 35 to 45 ml) in Fig. 49 isoelectric focussing was carried out in a thin-layer polyacrylamide gel on a Pharmacia flat-bed electrophoresis apparatus as described in Chapter 2. The sample used had to be concentrated 5 times. Fig. 60 shows that the material which was assumed to be the pure hydrophobic peptide was focussed as a single band with an estimated isoelectric point of 3.5.

4.2.1.3 A STUDY OF THE MATERIAL ELUTED LATE WITH WATER FROM THE PHENYL SUPEROSE COLUMN (FRACTIONS 36 TO 38 MIN IN FIG. 39)

Gel permeation chromatography

The material in the fractions that eluted late with water from the Phenyl Superose column (fraction 36 to 38 min in Fig. 39) were investigated in a similar manner to the material eluted early with water. A 100 µl sample of the concentrated material was mixed 1:1 with 0.2 mol/l Tris-HCl buffer pH 7, incubated for 2 h at 37°C, then chromatographed on a Superose 12 column and eluted with 0.1 mol/l Tris buffer. The material in this peak eluted at the bed volume and the profile was similar to that shown in Fig. 52.



1 2 3 4 5 6 7

Fig. 59: SDS-PAGE under reducing conditions of the fractions in the peaks from Figs. 48 and 49 on a high density 20% gel . Lane 1: bacitracin (1.45 kDa). Lane 2: cytochrome (12.5 kDa). Lane 3: fractions 35 to 45 ml in Fig. 49. Lane 4: fractions 30 to 45 ml in Fig. 48. Lane 5: fractions 30 to 45 ml in Fig. 48. Lane 6: fractions 45 to 75 ml in Fig. 48 Lane 7: fractions 45 to 75 ml in Fig. 48.

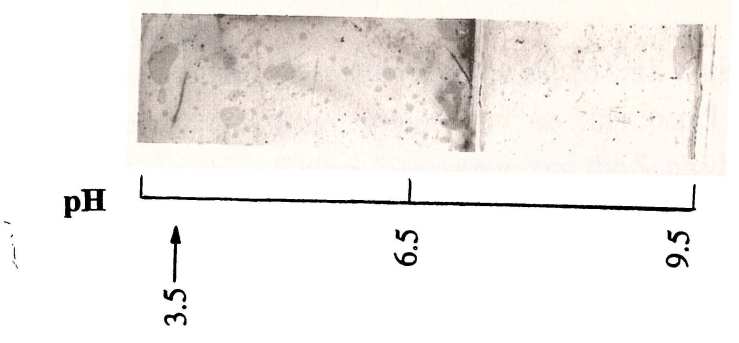


Fig. 60: Isoelectric focussing of fractions 35 to 45 ml in Fig. 49.

To confirm the molecular weight and the aggregation capacity of this material, it was chromatographed on the Sephadex G25 column. Accordingly, the Sephadex G25 column was equilibrated with 0.1 mol/l Tris-HCl, pH 7. A 250 µl sample of the material eluted late with water (fraction 36 to 38 in Fig. 39) was mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7, incubated for 2 h at 37°C, then chromatographed on a Sephadex G25 column and eluted with 0.1 mol/l Tris-HCl, pH 7. The results in Fig. 61 show a minor peak at the void volume (fractions 35 to 45 ml) and a larger peak at the bed volume (fractions 80 to 100 ml). It appeared that the material eluted from the Phenyl Superose column in fractions 36 to 38 was comprised predominantly of components with an estimated molecular weight of 1 kDa which failed to reaggregate, as well as a small portion of the component with molecular weight 5 kDa or over (which were probably components overlapping from the fractions eluted early with water from the Phenyl Superose). The fractions comprising each of the peaks in Fig. 61 (35 to 45 ml and 80 to 100 ml) were pooled, freeze-dried and dissolved in 125 µl of 0.1 mol/l Tris-HCl, pH 7.

Isoelectric focussing of the material in the peaks from Fig. 61

Isoelectric focussing was carried out in a thin-layer polyacrylamide gel on a Pharmacia flat-bed electrophoresis apparatus as described in Chapter 2. Samples from the concentrates of the fractions comprising each of the peaks in Fig. 61 (35 to 45 ml and 80 to 100 ml) mentioned above were applied onto the gel using filter paper saturated with the samples under test. Material in the void volume peak (35 to 45 ml) was focussed in three bands in the acidic region with estimated isoelectric points of 3.5 to 4 (Fig. 62). The material in the bed volume peak (80 to 100 ml) focussed in one band with an estimated isoelectric point of 3.5 similar to that seen in Fig. 60 from the isoelectric focussing of fractions 35 to 45 ml in Fig. 49.

Summary and conclusion:

As the previous section showed, the material which eluted with water from the chromatography on the Phenyl Superose column of the isopropanol supernatant of seminal plasma was a complex. Further fractionation on the Sephadex columns produced components of low molecular weight that varied in their anti-sperm activity; some with low and others with high levels of anti-sperm activity. Fractions below 5 and above 1.5 kDa showed anti-sperm activity higher than the fraction of high molecular weight which they originated from. The fractionation on Sephadex under disaggregating conditions also produced a pure hydrophobic peptide with an estimated molecular weight of 1 to 2 kDa and a pI of 3.5. This peptide showed no anti-sperm activity.

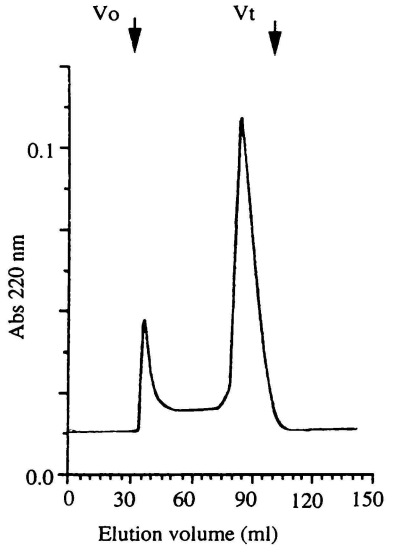


Fig. 61: Gel permeation chromatography of fractions 36 to 38 ml in Fig. 39.
Column: Sephadex G25SF.
Sample: 0.5 ml of the mixture of the concentrate of fractions 36 to 38 ml in Fig. 39 with 0.2 mol/l Tris-HCl, at pH 7.
Eluent: 0.1 mol/l Tris-HCl buffer, pH 7.
Flow rate: 1 ml/min.

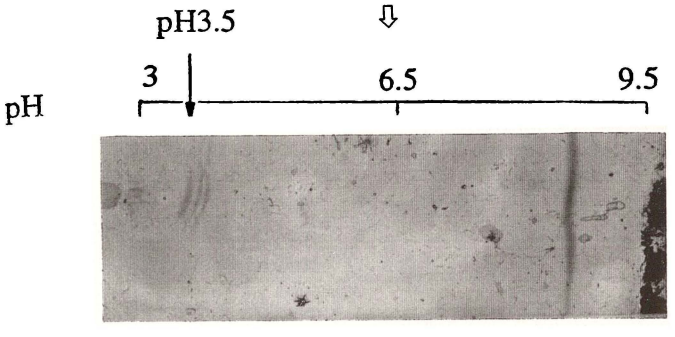


Fig. 62: Isoelectric focussing of the void volume peak (fractions 35 to 45 ml in Fig. 61).

The hydrophobic material resulting from the chromatography on Phenyl Superose was an aggregate which retained anti-sperm activity. This aggregate seems to be rich in anionic components.

4.2.1.4 A STUDY OF THE MATERIAL ELUTED WITH ACETONITRILE GRADIENT FROM THE PHENYL SUPEROSE COLUMN (FRACTIONS 54 TO 60 MIN IN FIG. 39)

Some material which bound very strongly to the Phenyl Superose column was eluted with a gradient of acetonitrile. This material was assumed to be very hydrophobic.

Test for anti-sperm activity

To determine if the material which eluted with acetonitrile from Phenyl Superose (fractions 54 to 60 min in Fig. 39) could affect the motility of washed sperm, a test for anti-sperm activity was conducted as previously described.

A 1 ml sample of the concentrate of fractions 54 to 60 min in Fig. 39 (Section 4.2.1) was mixed 1:1 with DS14G diluent. Sample from the fractions 24 to 36 min in Fig. 39 (material eluted early with water from Phenyl Superose) were included for comparison. Aliquots of 100 µl from the prepared samples were added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 19.

Table 19. Effect of the fractions in the peaks from the chromatography on Phenyl Superose shown in Fig. 39 on the motility of washed bull sperm.
The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		83.3±1.7^a	73.3±1.7^a
Material eluted with acetonitrile in Fig. 39	0.09	83.3±1.7^a	73.3±1.7^a
Fractions 24 to 36 min in Fig. 39	0.55	78.3±1.7^b	58.3±3.3^c
DSP*	3.63	83.3±1.7^a	65.0±2.9^b

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison.*
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

As can be seen, the component which eluted with acetonitrile from Phenyl Superose showed no reduction in the percentage of the motile sperm. Other fractions were included for comparison. While the material eluted early with water (fraction 24 to 36 min) and the retentate of dialysed seminal plasma significantly reduced the percentage of motile sperm after 18 h incubation, the material eluted with the acetonitrile in Fig. 39 did not reduce the percentage of motile sperm after 18 h incubation.

Gel permeation chromatography under neutral conditions

To determine its composition and its aggregating capacity, 0.5 ml of the concentrate of the fractions eluted with the acetonitrile gradient (54 to 60 min in Fig. 39, concentrated as described in Section 4.1.2.1) was mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7 and incubated at 37°C for 2 h. A 100 µl sample was then chromatographed under neutral conditions on the Superose 12 column and eluted with 0.1 mol/l Tris-HCl, pH 7. The result of the chromatography showed that no aggregation occurred. The material eluted at the bed volume of the Superose 12 column, with an estimated molecular weight of 1 kDa. The result was identical to that in Fig. 52 which was obtained with fractions 35 to 45 from Fig. 49 (a free anionic peptide with an estimated molecular weight of 2 kDa and a pI 3.5). Upon chromatography on Sephadex G25 (under neutral conditions) in a similar manner to that performed with the material eluted late with water from the Phenyl Superose column, this material which eluted with acetonitrile from Phenyl Superose column, eluted in one peak at the bed volume, with an estimated molecular weight of 1 to 1.5 kDa.

Isoelectric point determination

The isoelectric point of fractions 54 to 60 min from Fig. 39 was determined following the procedure previously described and the result was identical to that shown in Fig. 60; the material focussed in one band with an estimated pI of 3.5.

4.2.1.5 AMINO ACID ANALYSIS OF THE FRACTIONS FROM THE CHROMATOGRAPHY SHOWN IN FIGS. 39, 48 AND 49

Amino acid analysis of the fractions shown in Figs. 39, 48 and the isolated free anionic peptide shown in Fig. 49 was carried out as described in Chapter 2. The results are in Table 20. The Table shows that the isolated free peptides are acidic in nature. This confirmed the result from the isoelectric focussing determination. They are rich in aspartate and glutamate (but these could have been asparagine and glutamine before hydrolysis) as well as hydrophobic amino acids. The active fraction which was isolated after separation on Phenyl Superose appeared to contain basic

amino acids and is also rich in hydrophobic amino acids which explains why it bound to the Phenyl Superose column.

Table 20. Amino acid composition of the peptide (fraction 35 to 45) in Fig. 49, components eluted with acetonitrile in Fig. 39 (fractions 54 to 60 min) and fraction 45 to 75 ml in Fig. 48.

	Fractions 35 to 45 ml in Fig. 49	fractions eluted with acetonitrile in Fig. 39	fractions 45 to 75 ml in Fig. 48
Amino acid	**% of total**	**mol % of total**	**mol % of total**
Aspartate	15.4	13.9	7.8
Glutamate	9.8	11.9	10.4
Serine	7.7	6.2	6.0
Glycine	7.6	7.0	7.3
Histidine	2.2	2.3	1.9
Threonine	4.1	4.3	6.0
Alanine	6.0	6.1	6.2
Proline	8.6	9.8	9.9
Tyrosine	6.0	6.5	8.5
Valine	5.2	8.8	2.9
Methionine	0.0	0.0	0.0
Cystine+Cystein	0.0	0.0	0.0
Isoleucine	8.1	7.5	9.1
Leucine	9.8	8.4	6.1
Phenylalanine	9.3	7.3	6.0
Arginine	0.0	0.0	6.0
Lysine	0.0	0.0	5.5

4.2 DISCUSSION

An attempt was made in this Chapter to disaggregate the large molecular weight protein aggregates in seminal plasma by interfering with the ionic and hydrophobic attraction. Isopropanol precipitation under acidic conditions and immobilisation on a hydrophobic interaction column (Phenyl Superose) was partially successful in disaggregating the high molecular weight aggregates.

Although partial disaggregation was achieved with isopropanol precipitation, chromatography of the isopropanol supernatant and the sediment under neutral conditions showed that precipitation alone was not successful in disaggregating the high molecular weight aggregate to its components. The material retained the ability to form aggregates of high molecular weight despite attempts to break down the ionic and the hydrophobic interactions. However, relatively low molecular weight components were evident in the profile from the chromatography (Figs. 36 and 37) since most of the protein eluted in the region of molecular weight less than 20 kDa under neutral conditions.

Although the supernatant and the sediment from the isopropanol precipitation of seminal plasma appear to contain components of various molecular weights, anti-sperm activity was demonstrated in all fractions. The activity was not distinctly separated in any fraction after this partial disaggregation and it was associated with the aggregates of various molecular weights. The degree of anti-sperm activity of the components was different, however. Although the activity was present in all fractions, in a given fraction the activity was not necessarily present to the same extent. The anti-sperm activity was higher in the supernatant and the DMSO-soluble precipitate from the supernatant compared with the sediment (Table 16). This variation in the activity is interesting. The partial disaggregation may have arranged the molecule in a way which increases the total anti-sperm activity.

The precipitation with isopropanol under acidic conditions caused the separation of the supernatant and the sediment into components which were more detrimental to sperm than the original seminal plasma was (Table 16). There was an overall increase in anti-sperm activity after fractionation of seminal plasma with isopropanol. It appears that the shift toward low molecular weight after disaggregation of the high molecular weight components is responsible for this increase in activity. While all fractions exert high anti-sperm activity, including the DMSO-soluble material from the supernatant, the combination of these fractions decreases the overall activity.

This may be due to reaggregation. However, anti-sperm activity remained higher than in unfractionated seminal plasma (Table 16). This could be due to the presence of the low molecular weight components which did not aggregate into macromolecular form. This observation supports the earlier observation in Section 3.4 that low molecular weight components brought about by the disaggregation process higher anti-sperm activity than the parent aggregate of high molecular weight.

The treatment of seminal plasma with isopropanol at pH 1.7 followed by immobilisation on the Phenyl Superose column (hydrophobic interaction column) using a high salt concentration successfully resolved the isopropanol supernatant into several fractions. This technique did cause some disruption to the forces holding the aggregates (Fig. 39).

The material, presumably hydrophilic in nature (*i.e.* the unbound fraction from Phenyl Superose), did not appear to be completely disaggregated. This fraction could then be disaggregated to a minor low molecular weight component (Fig. 43b) which then reaggregated to larger components (this is discussed in detail in Chapter Six).

The anti-sperm activity did not partition into either the unbound or the bound material from the Phenyl Superose since it was found in both. However, further fractionation of the unbound material on Sephadex G25 under acidic conditions produced components which varied in their anti-sperm activity, being highest in the fraction with apparent molecular weight less than 1.5 kDa. This variation in activity within the presumedly hydrophilic components is interesting. The reduction in activity of fractions with apparent molecular weight greater than 1.5 kDa (Table 17) compared with the parent fraction (*i.e.* the supernatant of isopropanol) may be due to their separation from the hydrophobic components (*i.e* material that bound to Phenyl Superose) and the activity probably requires the two components in a certain proportion. However, the fraction of molecular weight less than 1.5 kDa (Fig. 42) showed high anti-sperm activity that was not significantly different from the parent fraction (isopropanol supernatant) but was significantly different from seminal plasma, although this fraction had a significantly lower protein concentration. This fraction may be a small aggregate having all the components necessary for the anti-sperm activity in the right proportion. This observation also emphasises that anti-sperm activity is higher in the low molecular weight component in seminal plasma which are derived from less active high molecular weight aggregates.

The bound material eluted early with water from Phenyl Superose (presumably hydrophobic in nature) also appears to be a complex of components of various molecular weights. Further fractionation on a Sephadex column, under disaggregative conditions, resulted in the production of components with molecular weight greater than 1.5 kDa that are more detrimental to sperm motility as well as components of molecular weight less than 1.5 kDa with no effect on sperm motility (Table 18). This is added evidence that partitioning of the anti-sperm activity in the fractions is not uniform but it is enhanced in some fractions and absent in the others. Components of less than 1.5 kDa from the hydrophobic material (*i.e.* material bound to Phenyl Superose) did not exhibit any anti-sperm activity. On the other hand components of similar molecular weight from the hydrophilic material (*i.e.* unbound material) with estimated molecular weights of less than 1.5 kDa) were distinctly active on sperm. This may be due to the ability of these low molecular weight hydrophilic components to form aggregates (Fig. 44) which posses anti-sperm activity while the component from the hydrophobic material failed to reaggregate.

It was observed in the present study that after separation on the Phenyl Superose column, the unbound and the bound material often formed aggregates of different sizes from the parent aggregates. The hydrophilic material was removed from the aggregates in the seminal plasma by binding of the hydrophobic material to the column. When re-chromatographed on the Sephadex column more separation occurred, which may have resulted in disturbance of the ratios of the material comprising the aggregate. The same result was obtained after the re-chromatography of peaks eluted with water from the Phenyl Superose column. The hydrophobic components from the bound material, with apparent molecular weights less than 1.5 kDa, failed to reaggregate under neutral conditions while the hydrophilic components with apparent molecular weight less than 1.5 kDa from the unbound material successfully reaggregated.

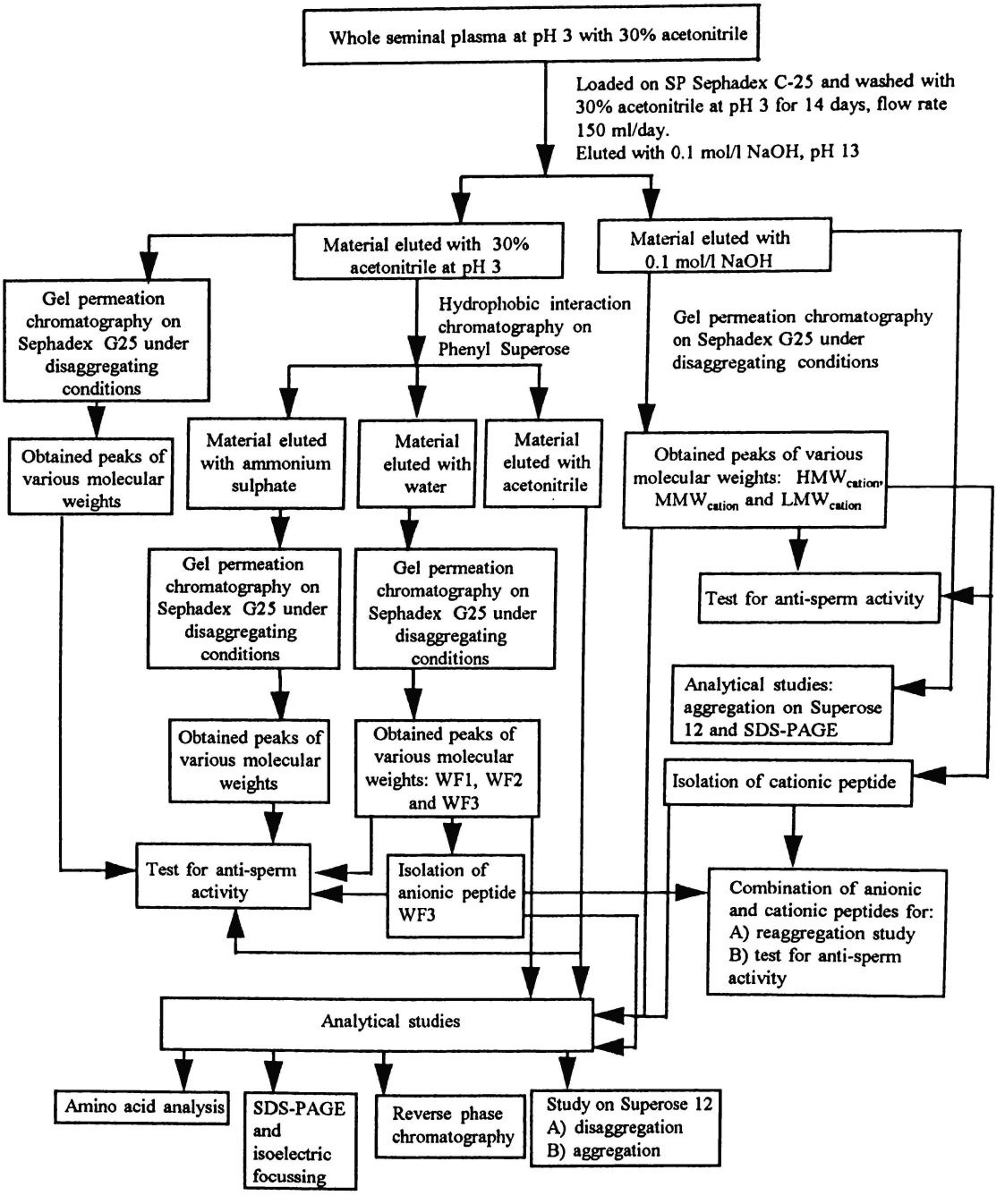
There has been an earlier attempt to separate the anti-sperm component from an active fraction of less than 20 kDa aggregate of seminal plasma on Phenyl Superose (Collingwood, 1990). The end result was a multitude of components that showed various degrees of reaggregation from 55 kDa to 11 kDa. A common aggregate of 14 to 16 kDa was found in all these fractions. The present study extended this concept and succeeded in further disaggregating the 14 to 16 kDa aggregate to smaller molecules and tested these for anti-sperm activity.

The technique of separation on Phenyl Superose successfully disaggregated the large molecular weight aggregate, at least partially, to produce a pure hydrophobic material which has a molecular weight less than 2 kDa and an isoelectric point of 3.5. The amino acid analysis of this pure peptide indicates that it is rich in acidic and hydrophobic amino acids with the absence of the basic amino acid. It appears that the activity is probably related somehow to the presence of the basic amino acids. The most active fraction contains a combination of basic, acidic as well as hydrophobic amino acids (Table 20).

Disaggregation in the presence of acetonitrile under acidic conditions was achieved resulting in a single peak at the bed volume of Superose 12 with an estimated molecular weight of 1 kDa. This peak at the bed volume could contain two components, one of them in a proportionally lower concentration than the other. This is an indication of complete disaggregation to the 1.2 kDa and 1 kDa components which were believed to comprise the large aggregates (Hameed, 1987). From the reaggregation results it seems that when the fraction had a high hydrophobicity with less cationic material in its composition, less aggregation occurred and the detrimental effect on sperm was reduced.

Acetonitrile appears to be useful in disaggregating the high molecular weight component as it interferes with the hydrophobic attraction. However, some small aggregates require high dilution to achieve complete disaggregation to the small peptides of estimated molecular weight of 1 kDa. Thus, dilution is also probably a major factor in the disaggregation process.

Flow chart for Section 5.1



CHAPTER FIVE

AN ATTEMPT TO ISOLATE THE CATIONIC COMPONENT

The work in Chapter 4 described attempts to disaggregate the large molecular weight aggregates in seminal plasma by disrupting the forces that contribute to their binding, such as ionic and hydrophobic attraction. By immobilisation of the hydrophobic component on a Phenyl Superose column it was possible to isolate the anionic peptide in a pure form but the cationic peptide remained in aggregated form, possibly with the other components. The aim in this Chapter was to isolate the cationic peptide from the native aggregate, characterise it and determine its effect on sperm motility .

5.1 AN ATTEMPT TO DISSOCIATE THE ANTI-SPERM ACTIVITY FROM THE AGGREGATE BY IMMOBILISATION ON A CATION EXCHANGER

The combination of acidic conditions and acetonitrile appeared to be useful in disaggregating the high molecular weight aggregates. It was presumed that by immobilising the positively charged components on a cation exchanger with extended exposure to acetonitrile, the hydrophobic forces could be disrupted, thus effecting further disaggregation. The procedure was carried out under acidic conditions (pH 3) to reduce the ionic interaction between anionic and cationic components. Lower pH was not used because it is not recommended for the SP Sephadex C-25 gel.

The procedure was carried out using SP Sephadex C-25 gel with a starting eluent of 30% acetonitrile at pH 3. At this pH, the positively charged material will bind to the column allowing the acidic component(s) and other material to pass through the column. At pH 3 anti-sperm activity was shown to pass through a membrane of 3.5 kDa molecular weight cut-off. Another factor considered was that a long period of dialysis (1 week) was necessary to allow extensive disaggregation (Shannon *et al.*, 1987). Thus, the procedure on the ion exchanger was carried out for 14 days at a very slow flow rate (*i.e.* 150 ml/day).

To 10 ml of seminal plasma obtained from fresh whole semen, acetonitrile was added to a final concentration of 30% and the pH was adjusted to 3.0 with HCl. The seminal plasma was then loaded on the SP Sephadex C25 column (20 x 2.5 cm). The column was washed with 30% (v/v) acetonitrile at pH 3.0 at a slow flow rate (150 ml/day) at 4°C. After two weeks the pooled eluate containing the unbound material was neutralised and its volume reduced to 10 ml by rotary evaporation.

The material bound to the cation exchanger was eluted with 0.01 mol/l NaOH. The pH of the eluted material was immediately adjusted to 7, and the volume reduced to 10 ml by rotary evaporation at 37°C. Both concentrates were stored frozen until further use.

The procedure on the ion exchanger was repeated once more using 10 ml of seminal plasma and the resulting concentrates were stored frozen until further use.

5.1.1 A STUDY OF THE MATERIAL ELUTED FROM THE ION EXCHANGER WITH ACETONITRILE

Material that passed through the cation exchanger with acetonitrile was presumably anionic material. To determine the composition of this unretarded material, and whether extended exposure to acetonitrile under acidic conditions had released the anti-sperm peptide from the macromolecular weight aggregate, the material was subjected to further investigation.

5.1.1.1 GEL PERMEATION CHROMATOGRAPHY ON SEPHADEX

To separate the low molecular weight components, chromatography on Sephadex G25 under disaggregation conditions was conducted. The frozen concentrate (10 ml) of the material that was eluted with 30% acetonitrile at pH 3 from the cation exchanger was thawed and 5 ml of this was taken to dryness by rotary evaporation then redissolved in 4 ml of 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. This material was held for 2 h at 4°C, and 1 ml was then chromatographed on a column of Sephadex G25 eluted with 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. The chromatography was repeated three times. The elution profile is shown in Fig. 63. As can be seen, a peak appeared at the void volume and others at the lower molecular weight region. The appearance of these peaks of low molecular weight is an indication of disaggregation of high molecular weight aggregate(s). It is evident that the long exposure to acetonitrile under acidic conditions caused some disaggregation resulting in the low molecular weight component(s) with apparent molecular weights of less than 5 kDa. The fractions comprising each peak (fractions 30 to 45 ml, 45 to 65 ml, 65 to 85 ml and 85 to 110 ml) accumulated from four runs were pooled, the pH of each pool adjusted to 7 and the volume reduced to 2.5 ml (half the volume of the original sample). All fractions were then desalted on Sephadex G10 eluted with water, and concentrated by rotary evaporation to 2.5 ml.

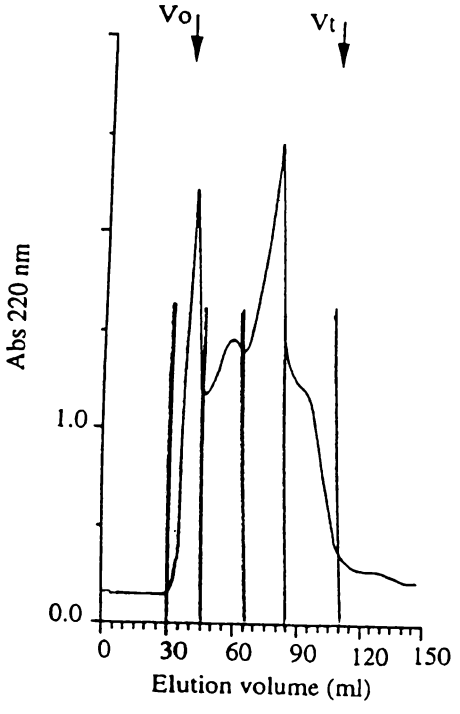


Fig. 63: Gel permeation chromatography of material eluted with the acetonitrile from the ion exchanger.
Column: Sephadex G25SF.
Sample: 1 ml of the concentrate in 30% acetonitrile in 0.02 mol/l HCl at pH 1.7.
Eluent: 30% acetonitrile in 0.02 mol/l HCl, pH 1.7.
Flow rate: 1 ml/min
Fraction volume: 5 ml

Test for anti-sperm activity in the fractions from Fig. 63

A 1 ml sample of the desalted concentrated fractions of each of the peaks from the chromatography on Sephadex shown in Fig. 63 was mixed 1:1 with DS14G diluent to be tested for anti-sperm activity. Aliquots of 100 µl were added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 21.

Table 21. Effect on motility of washed bull sperm of the fractions in the peaks from the chromatography on Sephadex G25 shown in Fig. 63.
The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		83.3±1.7^a	73.3±1.7^a
Fractions 30-45 ml in Fig. 63	0.38	81.7±1.7^{ab}	55.0±2.9^c
Fractions 45 to 65 ml in Fig. 63	0.50	78.3±1.7^b	40.0±2.9^d
Fractions 65 to 85 ml in Fig. 63	0.95	78.3±1.7^b	43.3±1.7^d
Fractions 85 to 110 ml in Fig. 63	0.39	76.7±1.7^b	41.7±1.7^d
Seminal plasma*	4.30	83.3±1.7^a	65.0±2.9^b

**Seminal plasma at 10% concentration for comparison*
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

Table 21 shows that all fractions from the chromatography on Sephadex G25 of the material not bound to the cation exchanger exhibited high anti-sperm activity. These components appeared to be more detrimental to sperm motility than the original seminal plasma. After 18 h incubation the low molecular weight components (fractions 45 to 65 ml, 65 to 85 ml, and 85 to 110 ml in Fig. 63) were significantly more active on sperm than either the high molecular weight components (fractions 30 to 45 ml) or seminal plasma. This finding was observed in the previous chapters where disaggregation of the macromolecular weight components resulted in the production of lower molecular weight components with higher anti-sperm activity than the fraction they originated from.

A disaggregation/aggregation investigation of the fractions from the chromatography on Sephadex G25 shown in Fig. 63

It is evident from the chromatography on Sephadex G25 (shown in Fig. 63) that disaggregation of the macromolecular aggregates had occurred. To determine whether

dilution with acetonitrile at pH 1.7 would result in further disaggregation, a series of dilutions with 30% acetonitrile of samples from the fractions comprising the peaks in Fig. 63 was conducted. The concentrate of the void volume peak (fractions 30 to 45 ml), the second peak (fractions 45 to 65 ml), the third peak (fractions 65 to 85 ml) and the fourth peak (fractions 85 to 110) in Fig. 63 all disaggregated to a single peak at the bed volume of the Superose 12 column with an estimated molecular weight of 1 kDa similar to that observed in Section 4.2.1.2 (Figs. 54a and b) when diluted 100 times.

To determine the composition of the components in the peaks from Fig. 63 and their ability to aggregate, the peaks were chromatographed on the analytical column of Superose 12 under neutral conditions. This was carried out on the concentrates of the fractions in the three peaks shown in Fig. 63 as described in Section 4.2.1.2, and resulted in a reaggregation of the components to a higher molecular weight similar to those seen in Figs. 50 and 51 when the anionic components was chromatographed in Section 4.2.1.2.

5.1.1.2 AN ATTEMPT TO DISAGGREGATE MATERIAL ELUTED WITH ACETONITRILE FROM THE ION EXCHANGER USING PHENYL SUPEROSE

The material eluted with acetonitrile at pH 3 from the ion exchanger was assumed to be made up of disaggregated components and be predominantly anionic in nature since it was unretarded at pH 3. Gel permeation chromatography of this material on Sephadex G25 under disaggregating conditions produced low molecular weight fractions of estimated molecular weight less than 5 kDa (*i.e.* the exclusion limit of Sephadex G25). However, these fractions reaggregated under neutral conditions. The eluate with acetonitrile from the ion exchanger was used as a starting material for chromatography on Phenyl Superose to generate more of the anionic peptide. This was based on the assumption that this material resulting from the dissociation procedure on the cation exchanger is rich in the anionic components and this makes it more attractive source for the isolation of the anionic peptide than the isopropanol supernatant of seminal plasma (Chapter 4). Accordingly, 5 ml sample of the concentrated unbound material which was eluted with acetonitrile from the cation exchanger was further concentrated to 2.5 ml by rotary evaporation. To this concentrate 2.5 ml of 3.4 mol/l ammonium sulphate was added to reach a final concentration of 1.7 mol/l. The solution, which appeared cloudy, was then centrifuged at 20 000 *g* for 30 min and the sedimented material was removed. A 1 ml portion of the clear supernatant was then loaded onto a Phenyl Superose column. Elution from Phenyl Superose was achieved by a descending gradient of 100% to 0%

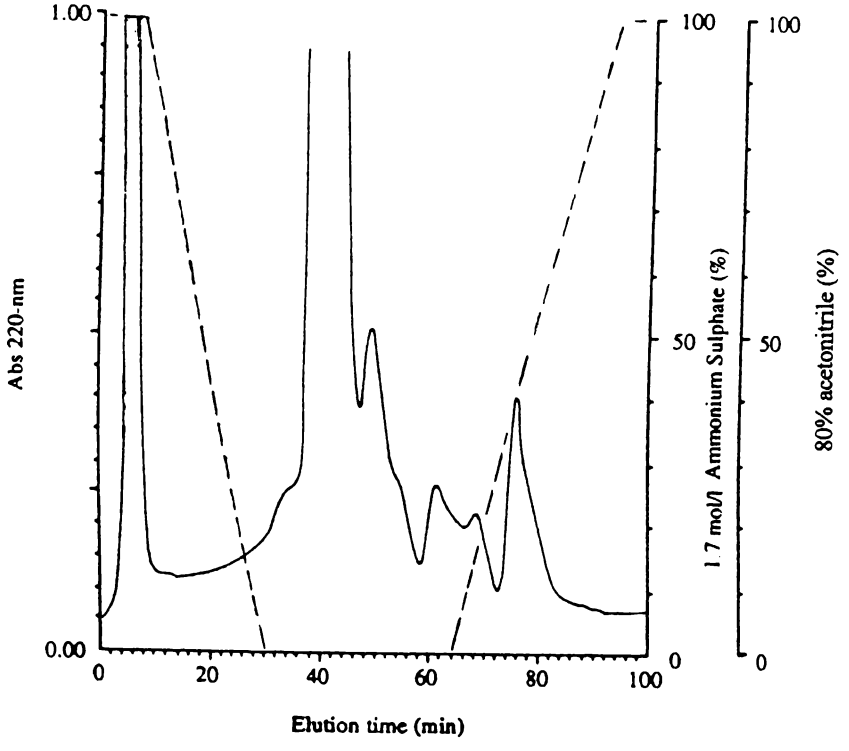


Fig. 64: Hydrophobic interaction chromatography of the unbound material eluted from the cation exchanger.
Column: Phenyl Superose HR 5/5 .
Sample: 1 ml of the concentrated material adjusted to 1.7 mol/l ammonium sulphate.
Eluents :
A) 1.7 mol/l ammonium sulphate
B) water
C) 80% acetonitrile
Gradient: 100% A for 10 min
0 to 100% B in 20 min
100% B for 34 min
0 to 100% C in 26 min
Flow rate: 0.5 ml/min.
Fraction volume: 0.5 ml.

of 1.7 mol/l ammonium sulphate followed by water, then an ascending gradient to 100% of acetonitrile as detailed in Fig. 64. Fractions of 0.5 ml were collected. The fractions from 2 to 10 min comprising the peak which eluted with ammonium sulphate (the material not bound to the Phenyl Superose), and fractions from 32 to 58 min, from 58 to 72 min, and from 72 to 84 min accumulated from 5 identical runs were pooled in their respective groups and the volume was reduced to 5 ml by rotary evaporation at 37°C.

A study of the peak which eluted with ammonium sulphate from Phenyl Superose (fractions 2 to 10 min in Fig. 64)

Gel permeation chromatography on Sephadex G25

Chromatography on Phenyl Superose resulted in separation of material that did not bind to the column indicating its hydrophilic nature and material that bound to the column indicating hydrophobicity. To determine if the material not bound to Phenyl Superose contained disaggregated low molecular weight components, 4 ml of the concentrated material (fractions 2 to 10 min) was rotary evaporated to dryness and the residue redissolved in 4 ml of 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. A 1 ml sample was then chromatographed on a column of Sephadex G 25 as detailed in Fig. 65. The chromatography was repeated three times. The elution profile in Fig. 65 shows that a peak eluted at the void volume indicating that the molecular weight was 5 kDa or over. Another peak eluted at the area of estimated molecular weight of 3.5 kDa and a third peak appeared at the bed volume of the Sephadex G25 column of estimated molecular weight less than 3.5 kDa. From the appearance of these peaks it seemed that disaggregation had occurred. The fractions comprising each peak in Fig. 65 from the four runs were pooled, the pH was adjusted to 7 and their volume was reduced to 2 ml. They were then desalted on Sephadex G10 eluted with water and their volume reduced to 2 ml (half of the volume of the sample chromatographed).

Test for anti-sperm activity

A 1 ml sample of each of the desalted concentrated fractions from Fig. 65 was diluted 1:1 with DS14G diluent to be tested for anti-sperm activity. Aliquots of 100 µl from the prepared samples were added to 900 µl of washed sperm suspended in 14G diluent to give a 10% concentration of the fraction and a sperm concentration of 10-12x10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 22.

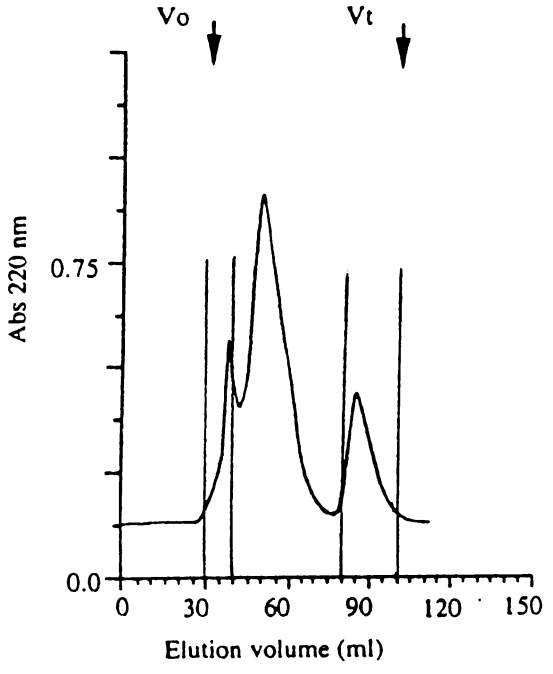


Fig. 65: Gel permeation chromatography of the peak eluted with ammonium sulphate from the chromatography on Phenyl Superose (fractions 2 to 10 min in Fig. 64).
Gel: Sephadex G25 SF
Sample: 1 ml of the concentrated material in 30% acetonitrile in 0.02 mol/l HCl at pH 1.7
Eluent: 30% acetonitrile in 0.02 mol/l HCl, pH 1.7
Flow rate: 1 ml/min
Fraction volume: 5 ml

Table 22. Effect of the fractions in the peaks from Fig. 65 on motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		83.3±1.7^{a}	73.3±1.7^{a}
Fractions 30 to 40 ml in Fig. 65	0.08	81.7±1.7^{a}	66.7±1.7^{b}
Fractions 40 to 80 ml in Fig. 65	0.42	83.3±1.7^{a}	70.0±2.9^{ab}
Fractions 80 to 100 ml in Fig. 65	0.19	80.0±2.9^{a}	61.7±1.7^{c}
DSP*	3.63	83.3±1.7^{a}	65.0±2.9^{bc}

**Retentate of seminal plasma dialysed against water at 10% concentration for comparison.*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The hydrophilic components which did not bind to the Phenyl Superose column reduced the percentage of motile sperm compared with the control after 18 h incubation, however, none of the fractions in Fig. 65 exerted high activity against sperm. It appears that separation of the hydrophilic from the hydrophobic components had affected the activity of some components in the peak eluted with ammonium sulphate. In the original sample (*i.e.* the material eluted with acetonitrile from the ion exchanger) fractions with similar apparent molecular weight exerted high activity against sperm.

Analytical study of the components in the ammonium sulphate peak

Chromatography on Sephadex G25 of the material that did not bind to the Phenyl Superose column showed the presence of low molecular weight components (Fig. 65). To determine the aggregating behaviour of this material under neutral conditions, it was chromatographed on an analytical column of Superose 12.

A 200 µl sample of the concentrate of the ammonium sulphate peak from the chromatography on Phenyl Superose (fractions 2 to 10 min in Fig. 64) was mixed 1:1 with 0.2 mol/l Tris-HCl at pH 7. A 400 µl sample of this solution was chromatographed on the analytical column of Superose 12 eluted with 0.1 mol/l Tris-HCl buffer at pH 7. The results showed material eluted in several peaks up to 80 kDa

similar to those shown in Fig. 40 (Section 4.2.1.1) indicating that the material was a complex of components.

Since the chromatography showed that the material was in aggregated form, a disaggregation study was conducted similar to that previously described. After a series of dilutions with 30% acetonitrile in 0.02 mol/l HCl at pH 1.7 , there was a shift in the molecular weight towards the bed volume of Superose 12. After 100 times dilution with 30% acetonitrile in 0.02 mol/l HCl at pH 1.7, the material disaggregated to one peak at the bed volume as shown in Fig. 66.

It was presumed that at least partial disaggregation to the small molecular weight peptide had taken place and that the third peak (fractions 80 to 100 ml) from the chromatography on Sephadex G25 shown in Fig. 65 might be one component. To check this possibility, 0.5 ml of the concentrated material of this peak was mixed 1:1 with 0.2 mol/l Tris-HCl buffer at pH 7 for 2 h at 37°C then chromatographed on a Sephadex G25 column and eluted with 0.1 mol/l Tris-HCl at pH 7 to see if they eluted in the same position. The results (in Fig. 67) show a large peak at the void volume indicating a molecular weight of 5 kDa or over, followed by other small peaks of lower molecular weight. It is clear that the material in the third peak from Fig. 65 has the ability to reaggregate to a higher molecular weight of more than 3.5 kDa. This component eluted at the bed volume of Sephadex G25 (Fig. 65) under disaggregating conditions (acetonitrile and acid). Once again this emphasises the forces of attraction that contribute to the formation of an aggregate.

A study of material eluted with water and acetonitrile from the chromatography on Phenyl Superose shown in Fig. 64

Material that bound to Phenyl Superose was presumably hydrophobic in nature. To determine whether chromatography on Phenyl Superose resulted in further disaggregation of the material that eluted with the acetonitrile from the cation exchanger, it was subjected to further studies.

Gel permeation chromatography of the material eluted early with water (fractions 35 to 60 in Fig. 64)

It was anticipated that disaggregation may had occurred after the chromatography on Phenyl Superose and as a result of that low molecular weight components may have been dissociated from the macromolecular aggregates. Chromatography on Sephadex G25 under dissociating condition was conducted to separate the low molecular weight from the high molecular weight components. Accordingly, 4 ml of the

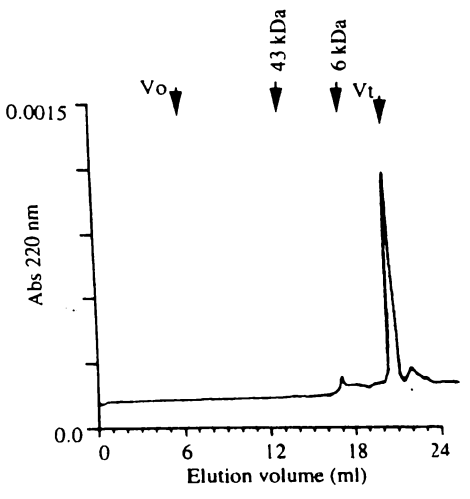


Fig. 66

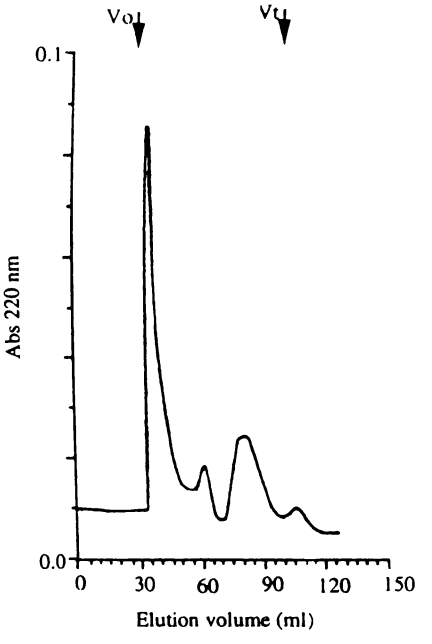


Fig. 67

Fig. 66: Gel permeation chromatography of material eluted with ammonium sulphate from the chromatography on Phenyl Superose (fractions 2 to 10 min in Fig. 64).
Column: analytical Superose 12 HR 10/30.
Sample: 200 µl of the sample diluted 100 times in 30% acetonitrile in 0.02 mol/l HCl at pH 1.7.
Eluent: 30% acetonitrile in 0.02 mol/l HCl at pH 1.7.
Flow rate: 0.5 ml/min

Fig. 67: Gel permeation chromatography of the material in the third peak in Fig. 65 (fractions 80 to 100 ml).
Gel: Sephadex G25 SF
Sample: 0.5 ml of the concentrated material mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7.
Eluent: 0.1 mol/l Tris-HCl, pH 7.
Flow rate: 1 ml/min

concentrated fractions that eluted early with water from the Phenyl Superose column (*i.e.* from 32 to 58 min in Fig. 64) was freeze-dried and redissolved in 4 ml of 30% acetonitrile in 0.02 mol/l HCl. The solution was held for 2 hours at 4°C, then 1 ml chromatographed on a column of Sephadex G25 eluted with 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. The chromatography was repeated three times. The results (in Fig. 68) show three peaks: one at the void volume (designated WF1) comprised of fractions 35 to 55 ml with a molecular weight 5 kDa or greater, followed by a second peak comprised of fractions 55 to 75 ml with a molecular weight <5 kDa and >1.5 kDa (designated WF2) and a third peak near the bed volume comprised of fractions from 75 to 100 ml with a molecular weight <1.5 kDa (designated WF3). The fractions comprising each of the peaks in Fig. 68 accumulated from four runs were pooled, their pH was adjusted to 7 and the volume of each of these fractions was reduced to 2 ml by rotary evaporation.

A 4 ml sample of the concentrated fractions that eluted late with water from Phenyl Superose (fractions 58 to 72 min in Fig. 64) were freeze-dried and the residue was redissolved in 4 ml 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. Chromatography of 1 ml of this solution on Sephadex G25 eluted with 30% acetonitrile in 0.02 mol/l HCl at pH 1.7 gave one peak near the bed volume of Sephadex G25 with an estimated molecular weight of less than 1.5 kDa (Fig. 69). The chromatography was repeated three times and the fractions comprising the peak from 70 to 95 ml accumulated from 4 runs were pooled, the pH was adjusted to 7 and the volume was reduced to 2 ml by rotary evaporation.

The fractions (72 to 84 min) eluted with acetonitrile from the chromatography on Phenyl Superose shown in Fig. 64 were treated in the same manner as with the fractions eluted with water. The result of the chromatography of this material on Sephadex G25 was similar to that shown in Fig. 69: one peak near the bed volume of Sephadex G25 with an estimated molecular weight of less than 1.5 kDa. The fractions comprising this peak were pooled, their pH was adjusted to 7 and the volume was reduced to 2 ml by rotary evaporation. This material was designated ACNfrac.

All fractions resulting from the chromatography on Sephadex G25 were desalted on Sephadex G10 eluted with water then concentrated to 2 ml by rotary evaporation. One ml of each fraction was then mixed 1:1 with DS14G to be tested for anti-sperm activity. The remainder of each fraction was kept for further investigation.

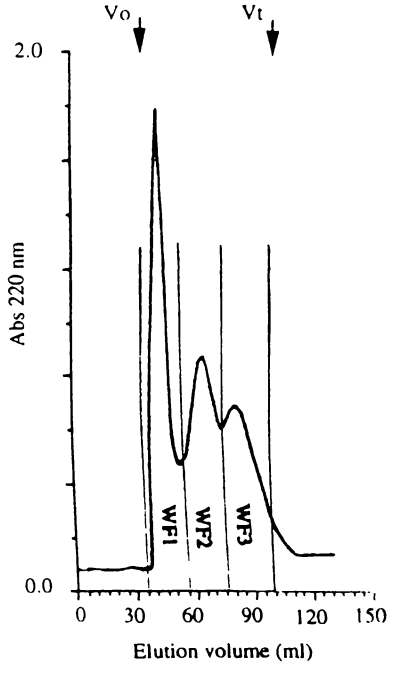


Fig. 68

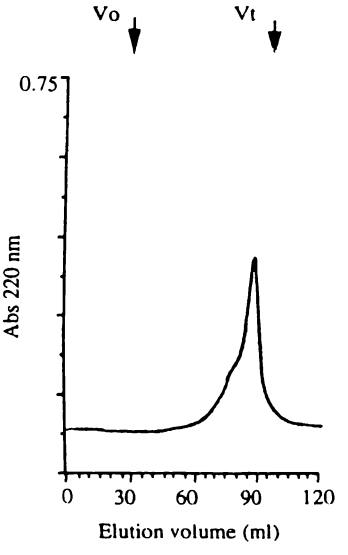


Fig. 69

Fig. 68: Gel permeation chromatography of the material that eluted early with water from the chromatography on Phenyl Superose (32 to 58 min in Fig. 64)
Column: Sephadex G25SF.
Sample: 1 ml of the freeze-dried fraction from Phenyl Superose dissolved in 30% acetonitrile in 0.02 mol/l HCl, pH 1.7.
Eluent : 30% acetonitrile in 0.02 mol/l HCl, pH 1.7
Flow rate: 1 ml/min.
Fraction volume: 5 ml.

Fig. 69: Gel permeation chromatography of the material that eluted late with water from the chromatography on Phenyl Superose (58 to 72 min in Fig. 64).
Gel: Sephadex G25 SF
Sample: 1 ml of the freeze-dried material dissolved in 30% acetonitrile in 0.02 mol/l HCl at pH 1.7.
Eluent: 30% acetonitrile in 0.02 mol/l HCl, pH 1.7.
Flow rate: 1 ml/min
Fraction volume: 5 ml.

Test for anti-sperm activity

Aliquots of 100 µl of the fractions under test were made up to 1 ml final volume with sperm suspension to give 10% (v/v) concentration of the fraction and 10-12 x 10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 hours incubation at ambient temperature (18 to 21°C). The results are shown in Table 23 and 24.

Table 23. Effect of the fractions shown in Fig. 68 on the motility of washed bull sperm

The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=4). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		82 0±2.0^a	71.3±1.3^a
WF1	0.517	82.5±1.4^a	54.0±2.4^c
WF2	0.370	72.5±1.4^b	39 0±2.5^d
WF3	0.220	84.0± 1.3^a	72.5±1.4^a
Seminal plasma*	4.30	80.0±2.0^a	65 ±2.0^b

**Seminal plasma at 10% concentration for comparison*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The results in Table 23 show that all but one of the fractions (WF3) reduced the motility of sperm after incubation. The high molecular weight fraction (WF1) did not decrease motility at first but showed marked effects on further incubation. WF2 slightly decreased motility soon after addition, and on further incubation showed a further decrease in the percentage of motile sperm. WF3 showed no more reduction in the percentage of motile sperm after 18 hours incubation than did the control.

Table 24. Effect of the fractions shown in Fig. 69 and ACNfrac on the motility of washed bull sperm

The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=4). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		82 0±2.0[a]	71.3±1.3[a]
Fractions 70 to 95 ml in Fig. 69	0.17	84 0±1.3[a]	71.3±1.3[a]
ACNfrac	0.15	82 0±2.0[a]	73.8±1.3[a]
Seminal plasma*	4.30	80 0±2.0[a]	65.0±2.0[b]

**Seminal plasma at 10% concentration for comparison*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The results in Table 24 indicate that the fractions in Fig. 69 (the original source being the material that eluted late with water from Phenyl Superose in Fig. 64) and ACNfrac (the original source being the material that eluted with the acetonitrile gradient in Phenyl Superose in Fig. 64) did not reduce the motility of washed sperm.

Analytical gel permeation chromatography of the material resulting from the chromatography on Sephadex column shown in Fig. 68 and 69

The material in the fractions that eluted early with water from Fig. 64 contained low molecular weight components as well as high molecular weight components as shown by the chromatography on Sephadex G25 under dissociating conditions. It was interesting that some of the fractions retained anti-sperm activity while some like WF3 and ACNfrac did not exhibit anti-sperm activity. Further investigation of the nature of these fractions was conducted.

Chromatography under neutral conditions

To determine the aggregating behaviour of the fractions from Fig. 68, 100 µl of the concentrate of WF1 was diluted 1:1 with 0.2 mol/l Tris-HCl buffer at pH 7, incubated for 2 h at 37°C, then chromatographed on a Superose 12 column eluted with 0.1 mol/l Tris-HCl at pH 7. The results in Fig. 70a showed a major peak near the bed volume with an estimated molecular weight of less than 20 kDa, and also several other peaks in the higher molecular weight area (up to 200 kDa) indicating that although partial disaggregation had taken place, the material was still a complex of components with various molecular weights.

When WF2 was subjected to similar study, the material in this peak which has an apparent molecular weight of less than 5 kDa under dissociating conditions appeared as a complex of components and reaggregated to a higher molecular weight. The results in Fig. 70b show several peaks with estimated molecular weights ranging from 5 to 200 kDa. The major peak appeared in the region above 20 kDa. However, the aggregation in both WF1 and WF2 was not complete: the original profile of seminal plasma was not obtained and the major peak in Fig. 70a and 70b was in the relatively low and medium molecular weight regions respectively (between 10 and 60 kDa).

When WF3 from Fig. 68, fractions 70 to 95 ml in Fig. 69, and ACNfrac were subjected to similar chromatographic procedures as for WF1 and WF2 there seemed to be no aggregation and a single peak eluted at the bed volume of the Superose 12 column. A representative chromatogram is shown in Fig. 71: the peak has an estimated molecular weight of 1 kDa.

Gel permeation chromatography under disaggregation conditions

The peaks in Fig. 68 which resulted from the chromatography on Sephadex G25 of the material that eluted early with water from the Phenyl Superose column were subjected to a disaggregative study as in Section 4.2.1.2. The high molecular weight components in WF1 and WF2 disaggregated to one peak at the bed volume of the Superose 12 column, with an estimated molecular weight of 1 kDa. The results of the study were similar to the results obtained in the disaggregative study (Figs. 54a and b) of the peaks eluted with water from chromatography on Phenyl Superose of the supernatant that resulted from precipitation of seminal plasma with isopropanol.

Reaggregation study of the combined fraction from the chromatography on Phenyl Superose (Fig. 64)

Although aggregation of the material in WF1 and WF2 occurred, aggregation to the high molecular weight components was not predominant. The origin of WF1 and WF2 was the hydrophobic material that bound to Phenyl Superose. The hydrophilic components that did not bind to Phenyl Superose also did not form full aggregation to the predominantly high molecular weight aggregates. To determine if some of the components necessary for aggregate formation passed through along with the hydrophilic material, the aggregation study was repeated by inclusion of a mixture of all fractions from the chromatography on Phenyl Superose shown in Fig. 64 (i.e. the hydrophilic and hydrophobic components). A 50 µl sample of the concentrate of the material which did not bind to the Phenyl Superose column (fraction 2 to 10 min in

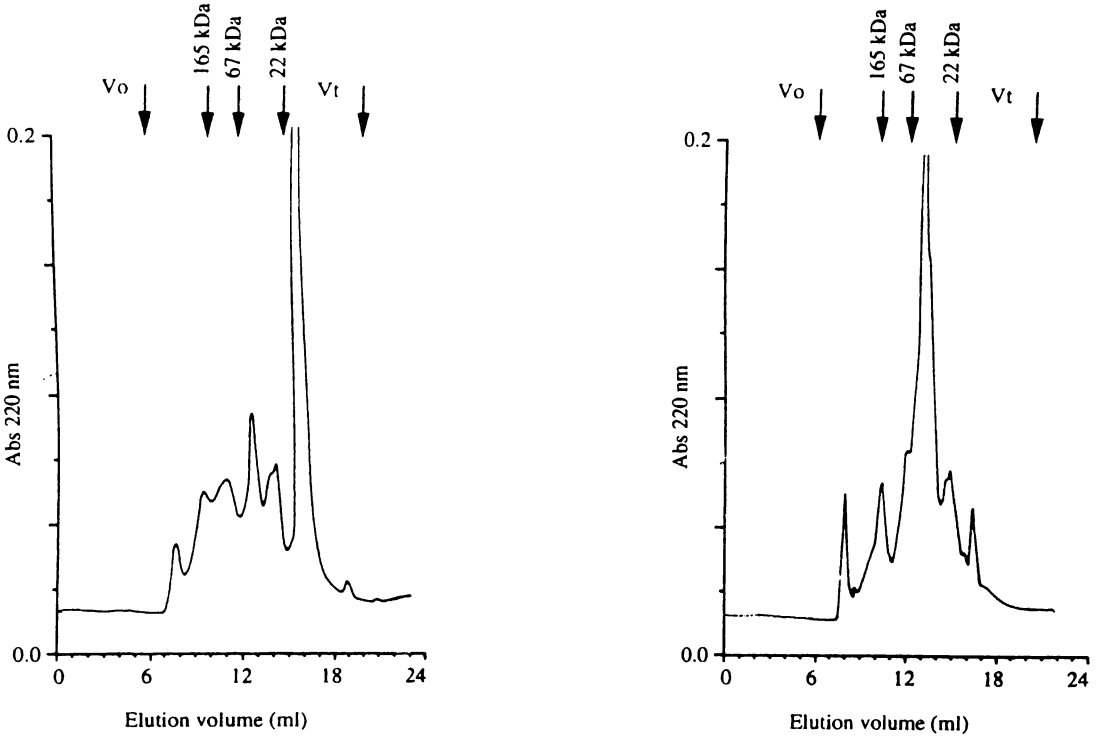


Fig. 70: Gel permeation chromatography of :
a) WF1 from Fig. 68
b) WF2 from Fig. 68
Column: Superose 12 (analytical grade, HR 10/30).
Sample: 200 µl of the mixture of the concentrate 1:1 with 0.2 mol/l Tris-HCl, pH 7.
Eluent: 0.1 mol/l Tris-HCl, pH 7.
Flow rate: 0.5 ml/min.

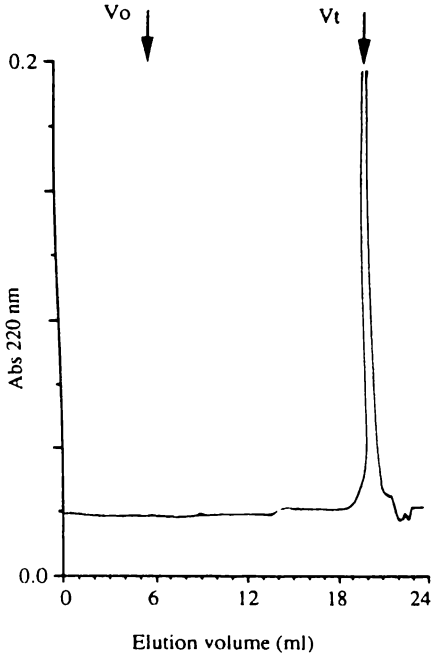


Fig. 71

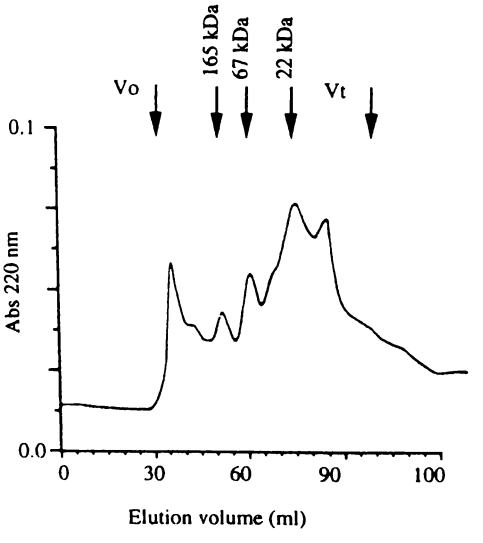


Fig. 72

Fig. 71: Gel permeation chromatography of WF3 from Fig. 68.
Column: Superose 12 (analytical grade, HR 10/30).
Sample: 200 µl of the mixture of the concentrate WF3 1:1 with 0.2 mol/l Tris-HCl, pH 7. at pH 7.
Eluent: 0.1 mol/l Tris-HCl pH 7.
Flow rate: 0.5 ml/min.

Fig. 72: Gel permeation chromatography of a combination of the bound (fractions 32 to 84 min and the unbound material (that passed through with the ammonium sulphate) to the Phenyl Superose column from Fig. 64.
Column: preparative Superose 12.
Sample: 400 µl of the mixture of the combined fractions 1:1 with 0.2 mol/l Tris-HCl, pH 7.
Eluent: 0.1 mol/l Tris-HCl pH 7.
Flow rate: 1 ml/min.

Fig. 64) was mixed with 50 µl samples of each of the three fractions concentrate of the material which bound to the Phenyl Superose column and was eluted with water and acetonitrile (*i.e.* fractions 32 to 58 min, 58 to 72 min, and 72 to 84). The combination was mixed 1:1 with 0.2 mol/l Tris-HCl, pH 7. After incubation for 2 hours at 37°C, the sample (400 µl) was chromatographed on a Superose 12 column eluted with 0.1 mol/l Tris buffer, pH 7.0. The results in Fig. 72 show that the components had the ability to reaggregate to the void volume (300 kDa or more). The original aggregate in seminal plasma (*i.e.* 500 and 250 kDa) was not the dominant one, probably because the proportion of the component is not right or due to continuous aggregation/disaggregation which might occur during gel permeation chromatography. Alternatively, it may be due to a concentration effect.

SDS-PAGE and isoelectric focussing of the fraction from Fig. 68

Gel permeation chromatography on Sephadex G25 of the material eluted with water from Phenyl Superose indicated the presence of low molecular weight components: one of the fractions (WF3) appeared to have a molecular weight of less than 1.5 kDa. However, chromatography on Superose 12 indicated a diversity of molecular weights in these fractions, although one failed to form an aggregate and appeared to have a molecular weight of less than 1.5 kDa (Fig. 71). To determine the molecular weight of these presumably anionic components, 1 ml samples of WF1, WF2 and WF3 were freeze-dried and redissolved in 200 µl 0.1 mol/l Tris-HCl, pH 7. They were then subjected to SDS-PAGE, under reducing conditions, on a Pharmacia PhastSystem. A 10 µl sample from each fraction was mixed with 10 µl sample buffer, and a 1 µl sample of each mixture was then loaded on a high density PhastGel (20% polyacrylamide gel).

They were then fixed and stained with 0.02% Coomassie R-250 according to the modification of Giulian *et al.* (1983 and 1985). The results are shown in Fig. 73. WF1 and WF2 under reducing conditions showed several bands with molecular weight below and above 6.5 kDa and both had a common bands in the region of 6.5 kDa. WF1 and WF2 showed also bands in the low molecular weight region below 5 kDa while WF3 showed one band with an estimated molecular weight of less than 2 kDa.

Since the origin of this material where these fractions came from had passed through the cation exchanger, it was assumed to be anionic in nature. To determine the charges of the low molecular weight fractions (less than 5 kDa), they were subjected to isoelectric focussing on a PhastSystem using a PhastGel IEF gel with a pH range of

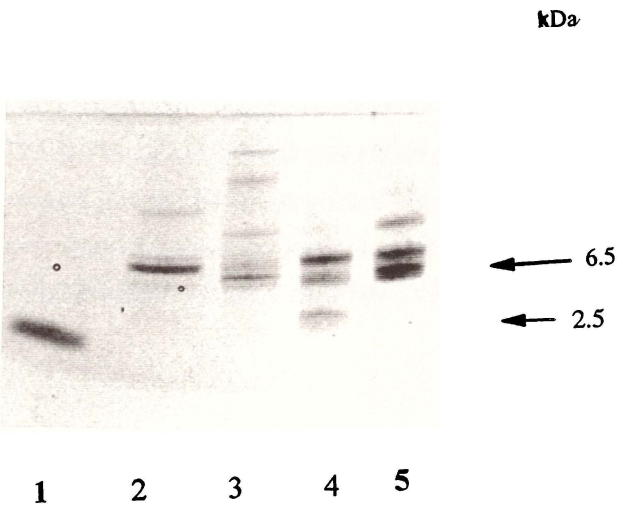


Fig. 73: SDS-PAGE under reducing conditions of the fractions from Fig. 68 on a 20% polyacrylamide gel.
Lane 1: WF3. Lane 2: aprotinin (6.5 kDa). Lane 3: WF1. Lane 4: WF2. Lane 5: WF1.

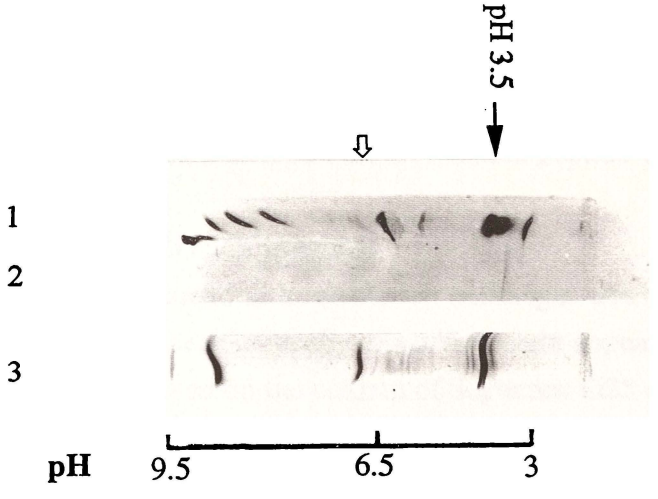


Fig. 74: Isoelectric focussing of fractions WF2 and WF3 from Fig. 68
Lane 1: standards pI 3 to 9.5. Lane 2: WF3. Lane 3: WF2.
⇨ shows point of sample application

3 to 9. A 1 µl sample of the concentrate mentioned above was loaded on the gel and the gel was stained with Coomassie blue as with the SDS-PAGE above. The results (in Fig. 74) showed that WF2 (the most active fraction) which was assumed to be anionic had several bands focussed in both the cationic and the anionic regions. More bands were seen in the anionic region with pI between pH 6.5 and 3. On the other hand, there was fewer bands in the basic region but they focussed in the highly basic area and one focussed at the edge of the gel indicating pI of 9.5 or probably more. This indicates that this fraction was an aggregate with a combination of cationic and anionic components. WF3 (a fraction that did not show anti-sperm activity) showed only one band which focussed in the anionic region with an estimated pI of 3.5. This finding is similar to the previous observation with regard to the free anionic peptide isolated previously from the chromatography on Phenyl Superose (Section 4.2.1.3, Fig. 60).

Reverse phase chromatography

In a further attempt to study the hydrophobic component, reverse phase chromatography was conducted to separate the species on the basis of hydrophobicity. Samples (0.25 ml) of WF1, WF2 and WF3 were rotary evaporated to dryness and each residue redissolved in 100 µl of 5 mmol/l tetrabutylammonium sulphate. The samples were chromatographed on the ProRPC reverse phase column as detailed in Fig. 75.

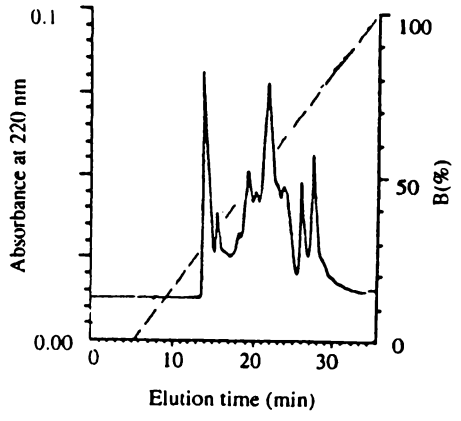
The material in WF1 and WF2 eluted in several peaks with the gradient. The separation shown in Fig. 75a and b indicated that the material was a complex of components with different degrees of hydrophobicity as some of them did not bind to the column. Fig. 75c shows the elution of WF3. This fraction was resolved as one peak confirming the results from the gel permeation chromatography, SDS-PAGE and isoelectric focussing which indicated that the isolated peptide was pure.

5.1.1.3 SUMMARY AND CONCLUSION:

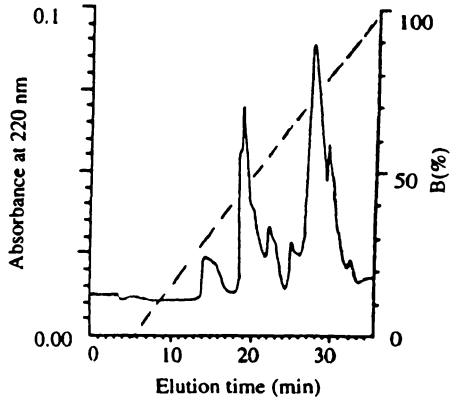
The anionic material which passed through the ion exchanger was still in an aggregated form, although a peak of small molecular weight appeared when these materials were chromatographed on the column of Sephadex G25 (Fig. 63) under disaggregating conditions. The disaggregated material was detrimental to sperm motility.

The anionic components which passed through the cation exchanger upon further separation on Phenyl Superose produced hydrophobic aggregates which were

(a)



(b)



(c)

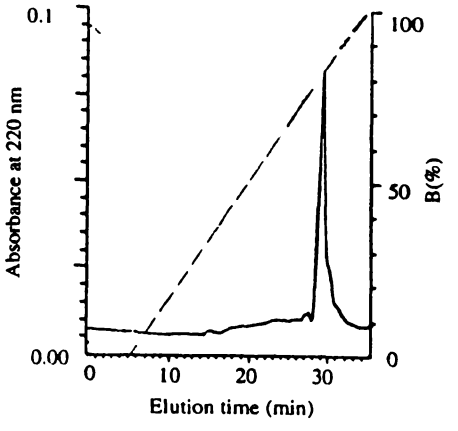


Fig. 75: Reverse phase chromatography of:
(a) WF1 (b) WF2 (c) WF3
Column: ProRPC HR 5/10
Sample: 100 µl of the freeze-dried fractions redissolved in 5 mmol/l tetrabutylammonium sulphate.
Eluent A: 5 mmol/l tetrabutylammonium sulphate
Eluent B: 80% acetonitrile in 5 mmol/l tetrabutylammonium sulphate.
Gradient: 0-100% B in 30 min

Flow rate: 0.5 ml/min

detrimental to sperm motility. However, a small hydrophobic anionic peptide resulting from the separation on Phenyl Superose did not affect sperm motility.

The hydrophobic nature of the protein(s) or peptide(s) was evident from the extent they bound to the column, and the greater the binding, the later they eluted with water. Although separation on a hydrophobic interaction column succeeded in separating the free anionic peptide, the hydrophilic component which passed through the column was still in an aggregated form.

Disaggregation of the aggregates by high dilution in acetonitrile gave a bed volume peak on the gel permeation chromatography. It is worth noting that when the disaggregative studies were done with a small factor of dilution, disaggregation could be observed for the individual peak(s), this was observed as a change in the size and the position of elution of the peak.

The failure of the components to reaggregate or to form large aggregates was probably due to that one of the components necessary for aggregate formation was missing or that the two components (*i.e.* cationic and the anionic) necessary to form this aggregate(s) were in the wrong proportions.

5.1 2 A STUDY OF THE MATERIAL THAT ELUTED FROM THE CATION EXCHANGER WITH NaOH

Material from seminal plasma that remained bound to the cation exchanger after extensive exposure to acetonitrile at pH 3 presumably is part of the aggregate which remains after this disaggregation procedure. If this was the case then this material should be freed from the anionic components which passed through the cation exchanger with the acetonitrile eluent. This material which eluted with NaOH from the cation exchanger was further investigated to determine its nature and composition, and its anti-sperm activity.

5.1.2.1 ANALYTICAL GEL PERMEATION CHROMATOGRAPHY UNDER NEUTRAL CONDITIONS

To further analyse the cationic material that remained bound to the cation exchanger after extended exposure to acid and acetonitrile and eluted with NaOH, it was subjected to chromatography on Superose 12 under neutral conditions. Accordingly, the frozen concentrate was thawed and 250 µl of this concentrate was mixed 1:1 with 0.2 mol/l Tris-HCl at pH 7, incubated for 2 h at 37°C then chromatographed on the Superose 12 column eluted with 0.1 mol/l Tris-HCl, pH 7. The results shown in Fig.

76 indicate that the cationic material was still made up of high molecular weight aggregates. Interestingly, most of the protein eluted at the high molecular weight area: a major peak eluted at the void volume of the column of Superose 12 with an estimated molecular weight of 300 kDa or greater. However, peaks of low molecular weight also occurred indicating partial disaggregation had occurred as a result of extended exposure and dilution with acetonitrile at pH 3. The cationic components do have the ability to form larger aggregates than those formed by the anionic components which passed through the cation exchanger column. The cationic material reaggregated up to the void volume of Superose 12 and the profile was similar to native seminal plasma with regard to the high molecular weight of the aggregates. Furthermore, the peak at the void volume peak was the dominant one.

SDS-PAGE of the fractions in the void volume peak from the chromatography shown in Fig. 76

The cationic components showed by gel permeation chromatography under neutral conditions to contain high molecular weight components that eluted mostly at the void volume region. To determine if the material in the void volume peak in Fig. 76 consists of one component or a complex of components, in the form of an aggregate, SDS-PAGE under reducing conditions was performed. The chromatography of the cationic material on Superose 12 was repeated as above and the fractions comprising the peak from 5 to 8 ml in Fig. 76 were pooled and freeze-dried. The freeze-dried material was redissolved in 100 µl of 0.1 mol/l Tris-HCl at pH 7. A 10 µl sample of the concentrate was mixed 1:1 with 5 times concentrated sample buffer and electrophoresed on a 12.5% polyacrylamide gel. The results (in Fig. 77) indicated the presence of high molecular weight components represented by the bands at the top of the gel with estimated molecular weights of 200 kDa or greater and several other major and minor bands of lower molecular weights between 97 and 14 kDa. Major bands in the relatively low molecular weight region are between 14 and 18 kDa were seen. This indicated that it was a complex of molecules since bands occurred in the region of molecular weight less than that estimated by the gel permeation chromatography, similar to that seen with the high molecular weight fraction of seminal plasma and whole seminal plasma, however, the major bands at the top of the gel have a molecular weight 200 kDa or greater. It is clear that although the elution profile showed a peak with molecular weight of 300 kDa or over, the electrophoresis in the presence of SDS showed disaggregation to lower molecular weights.

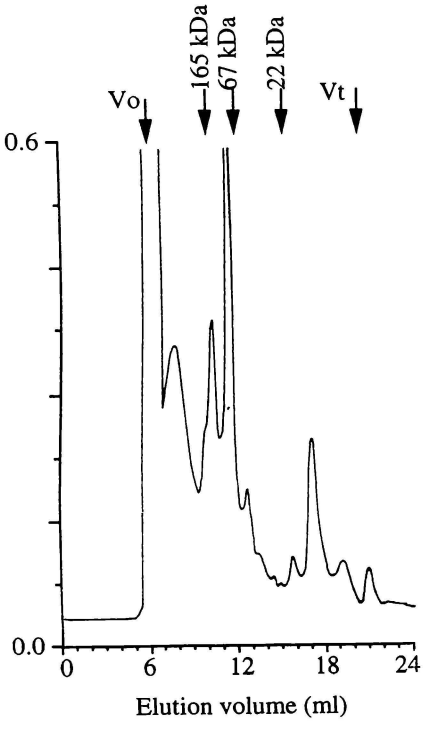


Fig. 76

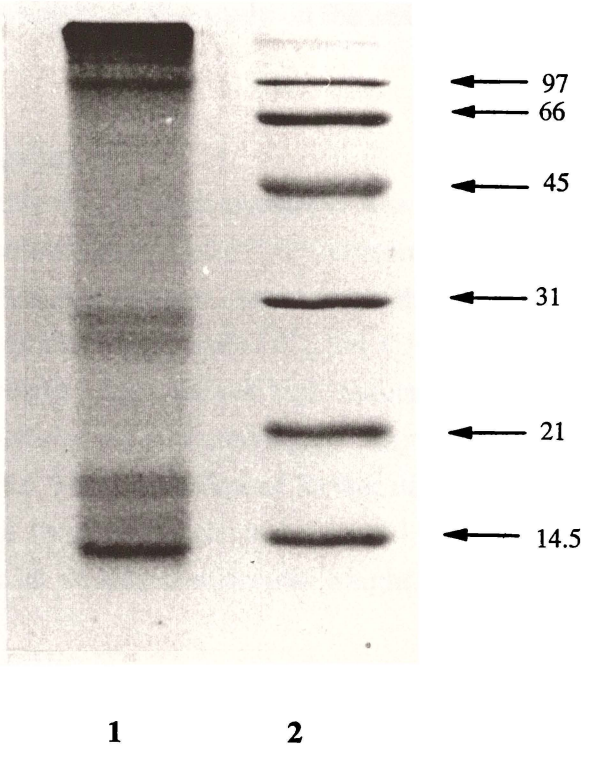


Fig. 77

Fig. 76: Gel permeation chromatography of the cationic material eluted with 0.1 mol/l NaOH from the cation exchanger.
Column: Superose 12 (analytical grade, HR 10/30).
Sample: 250 µl of the cationic material mixed 1:1 with 0.2 mol/l Tris-HCl, at pH 7.
Eluent: 0.1 mol/l Tris-HCl, pH 7.
Flow rate: 0.5 ml/min.
Fraction volume: 0.5 ml.

Fig. 77: SDS-PAGE, under reducing conditions, of the material in the void volume peak from Fig. 76 (fractions 5 to 8 ml) on a 12.5% polyacrylamide gel. Lane 1: fractions 5 to 8 ml in Fig. 76. Lane 2: molecular weight markers (97 to 14.5 kDa).

5.1.2.2 A DISAGGREGATING STUDY OF THE CATIONIC MATERIAL

Gel permeation chromatography and SDS-PAGE of the cationic material showed that this material is a complex with a wide range of molecular weight components. To determine if the high molecular weight components can be further disaggregated to the lower molecular weight components, it was subjected to a study on the analytical Superose 12 column.

Chromatography under disaggregating condition

A 100 µl sample of the concentrated NaOH eluate from the ion exchanger was rotary evaporated to dryness The residue was then redissolved in 1 ml of 30% acetonitrile in 0.02 mol/l HCl at pH 1.7. A 100 µl sample of this solution was chromatographed on the Superose 12 column eluted with 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. There was an extensive shift in the molecular weight. The peaks of high molecular weight seen in Fig. 76 disappeared and all the material eluted in the region below 20 kDa. The profile was similar to that shown in Fig. 54a. The effect of further dilution was even more drastic. A 100-fold dilution with 30% acetonitrile in 0.02 mol/l HCl at pH 1.7 resulted in a peak at the bed volume with estimated molecular weight of 1 kDa as shown in Fig. 78.

Reaggregation study of the disaggregated cationic material

Ten-fold dilution caused disaggregation and the cationic material eluted in several peaks of low molecular weight. To determine if this disaggregated material could reaggregate to a higher molecular weight the chromatography described above with the ten times diluted material was repeated ten times and the fractions 16 to 21 ml were pooled. The pooled fractions were rotary evaporated to dryness and redissolved in 100 µl of 0.1 mol/l Tris buffer at pH 7, held for 2 h at 37°C then chromatographed on the Superose 12 column eluted with 0.1 mol/l Tris-HCl, pH 7. The results in Fig. 79 show aggregation up to the void volume of Superose 12, though the major peaks were not the high molecular weight components as in Fig. 76. The disaggregated material thus had the capacity to reform aggregates of high molecular weight once the disaggregation conditions were removed.

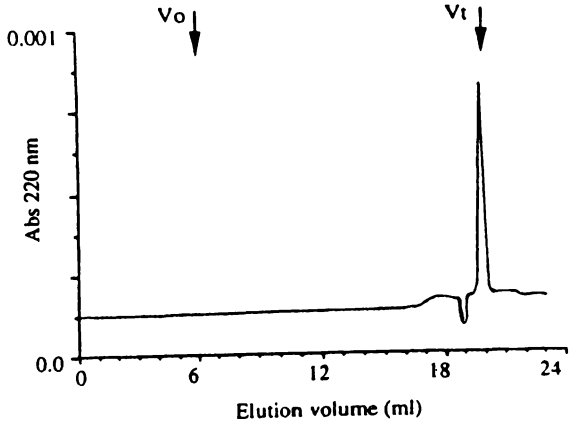


Fig. 78: Gel permeation chromatography of the cationic material eluted with NaOH from the cation exchanger.
Column: Superose 12 (analytical grade, HR 10/30).
Sample: 100 µl of the material diluted 100x in 30% acetonitrile in 0.02 mol/l HCl, at pH 1.7.
Eluent: 30% acetonitrile in 0.02 mol/l HCl, at pH 1.7.
Flow rate: 0.5 ml/min.

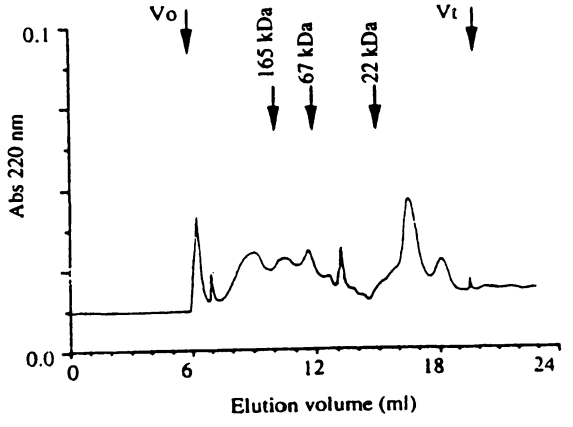


Fig. 79: Gel permeation chromatography of fractions 17 to 21 ml from the chromatography of the cationic material on Superose 12 as in Fig. 54a.
Column: Superose 12 (analytical grade, HR 10/30).
Sample: 100 µl of the dry material redissolved in 0.1 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl pH 7.
Flow rate: 0.5 ml/min.

5.1.2.3 PREPARATIVE GEL PERMEATION CHROMATOGRAPHY OF THE CATIONIC MATERIAL ON SEPHADEX G25 UNDER DISSOCIATING CONDITIONS

The analytical studies described above showed that material that bound to the cation exchanger and eluted with 0.1 mol/l NaOH appeared to be a complex of various molecular weight components. To separate the low molecular weight from the high molecular weight components it was subjected to chromatography on Sephadex G25 under disaggregative conditions. A 5 ml sample of the cationic material (Section 5.1) was freeze-dried and redissolved in 4 ml of 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. The cationic material was not completely soluble at pH 1.7 and hence was centrifuged at 20 000 g for 10 min. A 1 ml sample of the clear supernatant was chromatographed on the Sephadex G25 column eluted with 30% acetonitrile in 0.02 mol/l HCl, pH 1.7 as detailed in Fig. 80. The chromatography was repeated 3 times. The results show that the cationic material was resolved into three peaks: one at the void volume and two in the region after the void volume with estimated molecular weights <5 kDa (Fig. 80). These three peaks were designated: HMW_{cation} (fractions 30 to 45 ml), MMW_{cation} (fractions 45 to 70 ml) and LMW_{cation} (fractions 70 to 110). The fractions comprising each peak from Fig. 80 accumulated from 4 runs were pooled, the pH neutralised with NaOH, and their volume was reduced to 5 ml. It was then desalted on Sephadex G10 eluted with water. A 500 µl portion of each was retained for analysis and the remainder was freeze-dried.

The insoluble material at pH 1.7 was suspended in 5 ml of water and the pH was raised slowly. At pH 7 the material started to go into solution and it was completely soluble at pH 8.5. On readjusting the pH back to 1.7, precipitation occurred. The precipitated material was taken up with 50 mmol/l ammonium bicarbonate (pH 8.5) and was chromatographed on Sephadex G25 column and eluted with 50 mmol/l ammonium bicarbonate (pH 8.5). The material eluted at the void volume indicating a molecular weight of 5 kDa or greater. When this material was chromatographed on Superose 12 the elution profile indicated aggregation and peaks of molecular weight from 20 kDa up to the void volume occurred. This material appeared to be an aggregate and could not be processed at low pH. Accordingly, the study was carried out with the soluble material.

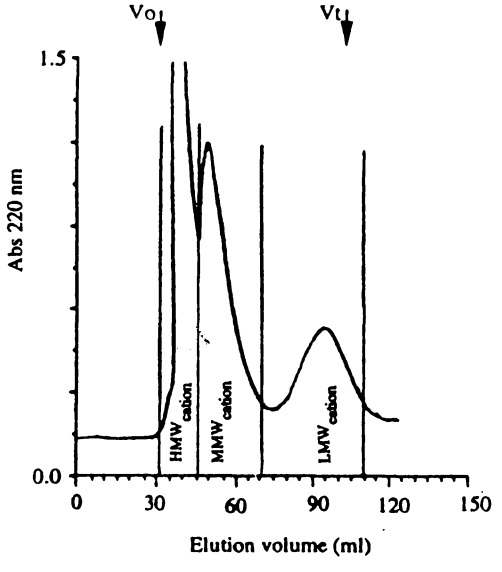


Fig. 80: Gel permeation chromatography of the cationic material eluted with NaOH from cation exchanger.
Column: Sephadex G25SF.
Sample: 1 ml of the cationic material in 30% acetonitrile in 0.02 mol/l HCl at pH 1.7.
Eluent : 30% acetonitrile in 0.02 mol/l HCl, pH 1.7.
Flow rate: 1 ml/min.
Fraction volume: 5 ml.

Test for anti-sperm activity of the fractions in peaks from the chromatography shown in Fig. 80

Freeze-dried fractions from Fig. 80 were reconstituted in 4.5 ml 14G buffer to be tested for anti-sperm activity. Aliquots of 100 µl of the fractions under test were made up to 1 ml final volume with sperm suspension in 14G to give 10% (v/v) concentration of the fraction and 10-12 x 10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 25.

Table 25. Effect of the fractions shown in Fig. 80 on the motility of washed sperm.

The values for motility are the percentage of motile sperm immediately and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=4). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		81.3±2.4^{a}	72.3±3.2^{a}
HMW_{cation}	0.52	76.3±3.8^{ab}	37.5±3.2^{c}
MMW_{cation}	0.26	72.5±3.3^{b}	59.0±3.8^{b}
LMW_{cation}	0.11	71.3±2.4^{b}	34.0±5.6^{c}
Seminal plasma*	4.30	81.3±2.4^{a}	65.0±5.8^{ab}

**Seminal plasma at 10% concentration for comparison.*
Like superscripts within a column indicate no significant difference between means ($p<0.05$).

Addition of the MMW_{cation} and LMW_{cation} fractions immediately reduced the percentage of motile sperm compared with the control. Incubation at ambient temperature for 18h reduced the number of motile sperm with all fractions, quite drastically in the case of HMW_{cation} and LMW_{cation}. Washed sperm were hyperactivated after the additions of the fractions and showed curved tails when observed after 18 h incubation. The relative amount of protein in the fractions tested differed, and in LMW_{cation} was significantly less than in HMW_{cation}. The anti-sperm activity of the HMW_{cation} and LMW_{cation} was significantly higher than that of the original seminal plasma that these fractions were derived from. This clearly indicates the role of the aggregates in seminal plasma in attenuating the effects of the damaging components.

SDS-PAGE of the fractions in the peaks from the chromatography on Sephadex G25 shown in Fig. 80

The cationic fractions exhibited high anti-sperm activity although this varied amongst the fractions. It was interesting to see whether disaggregation on Sephadex G25 had generated components freed from the aggregate or whether it still existed as a complex. To have a better estimate of their composition and their molecular weight, SDS-PAGE on a 20% high density polyacrylamide gel under reducing conditions was performed. A 100 µl sample of each of the concentrates of HMW_{cation}, MMW_{cation} and LMW_{cation} were freeze-dried and redissolved in 25 µl 0.1 mol/l Tris-HCl, pH 7. A 10 µl sample of each was then mixed 1:1 with a 5 times concentrated sample buffer and a 1 µl sample of each mixture was loaded on the gel. Electrophoresis was performed on the PhastSystem. The results in Fig. 81 show that HMW_{cation} and MMW_{cation} contain several components with molecular weights ranging between 3 and 12 kDa and greater, while the LMW_{cation} gave one band with an estimated molecular weight less than 3 kDa. Thus, the HMW_{cation} and MMW_{cation} were still a complex of components indicating an aggregate while the LMW_{cation} appeared to be one component. The results also showed that the MMW_{cation}, which appeared to have a molecular weight less than 5 kDa from the chromatography on Sephadex G25 under disaggregating conditions (Fig. 80), was a complex containing components higher than 5 kDa since bands appeared in the region of molecular weight much higher than 5 kDa.

Further study of the HMW_{cation}

Isoelectric focussing of the HMW_{cation}

The void volume peak in Fig. 80 (HMW_{cation}) separated into a wide range of molecular weights upon SDS-PAGE. To determine whether the components in this complex were cationic, isoelectric focussing was performed on a PhastSystem using a PhastGel IEF gel with a pH range of 3 to 9. A 1 µl sample of the concentrate of the HMW_{cation} was loaded onto the IEF-gel. The HMW_{cation} separated into several bands focussed in both the anionic and cationic regions as well as in the neutral region (Fig. 82). Bands at the edges of the gel were observed indicating extreme pI (*i.e.* very anionic and cationic respectively). Clearly, this complex of components was not homogenous with regard to the charges.

Reverse phase chromatography of the HMW_{cation}

In a further attempt to study the cationic components, reverse phase chromatography was conducted to separate the species on the basis of hydrophobicity. A 0.25 ml

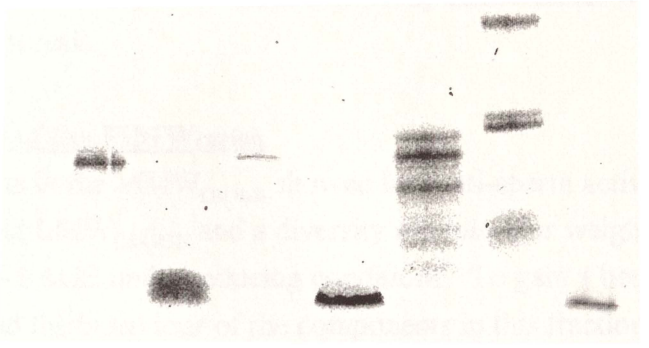


Fig. 81. SDS-PAGE of: HMW_{cation}, , MMW_{cation} and LMW_{cation} on a high density PhastGel with a continuous separating gel zone of 20% polyacrylamide under reducing conditions. Lane 1: cytochrome c (12.5 kDa). Lane 2: insulin B chain (3.4 kDa). Lane 3: cytochrome c (12.5 kDa). Lane 4: bacitracin (1.45 kDa). Lane 5: MMW_{cation}. Lane 6: HMW_{cation}. Lane 7: LMW_{cation}.

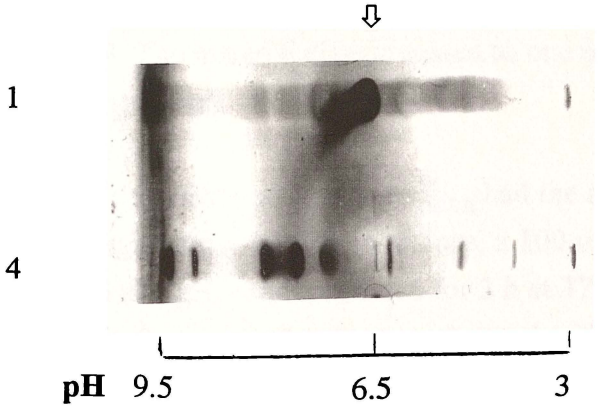


Fig. 82. Isoelectric focussing of fractions HMW_{cation}.

Lane 1: HMW_{cation}. Lane 2 and 3: irrelevant. Lane 4: standards pI 3 to 9.5.

⇨ shows point of sample application

sample of the concentrate of HMW_{cation} (Fig. 80) was rotary evaporated to dryness and the residue was redissolved in 100 µl of 0.1% trifluoroacetic acid. The sample was chromatographed on the reverse phase column as detailed in Fig. 83.

The material in HMW_{cation} eluted in several peaks with the gradient. The separation shown in Fig. 83 indicated that the material was a complex of components with different degrees of hydrophobicity as some of them did not bind to the column. This was not surprising since the HMW_{cation} was shown by isoelectric focussing to have anionic and cationic components, and a diversity of molecular weight from the results of the electrophoresis.

Further study of the MMWcation

The components in the MMW_{cation} showed less anti-sperm activity than the HMW_{cation} and LMW_{cation} and a diversity of molecular weights higher than 5 kDa upon SDS-PAGE under reducing conditions. To gain a better idea about the composition and the behaviour of the components in this fraction it was subjected to further analysis.

Gel permeation chromatography of MMW_{cation} under disaggregating/aggregating conditions

To determine whether the material in the second peak in Fig. 80 (MMW_{cation}) could disaggregate it was subjected to chromatography on Superose 12 under disaggregating conditions. A 10 µl sample from the concentrated material of MMW_{cation} was diluted 100 times in 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. A 100 µl sample was chromatographed on the Superose 12 column eluted with 30% acetonitrile in 0.02 mol/l HCl, pH 1.7. The result of the chromatography was similar to that seen in Fig. 78. The material disaggregated to one peak at the bed volume with an estimated molecular weight of 1 kDa.

To determine whether the material in MMW_{cation} had the ability to form an aggregate of high molecular weight under neutral conditions, a 100 µl sample was mixed 1:1 with 0.02 mol/l Tris-HCl, pH 7 and incubated for 2 h at 37°C. It was then chromatographed on a Superose 12 column and eluted with 0.1 mol/l Tris-HCl, pH 7. The material in this fraction appeared to have the ability to reaggregate. Although reaggregation to a high molecular weight component of up to 80 kDa occurred, the major peak was near the bed volume with estimated molecular weight between 5 and 15 kDa (Fig. 84).

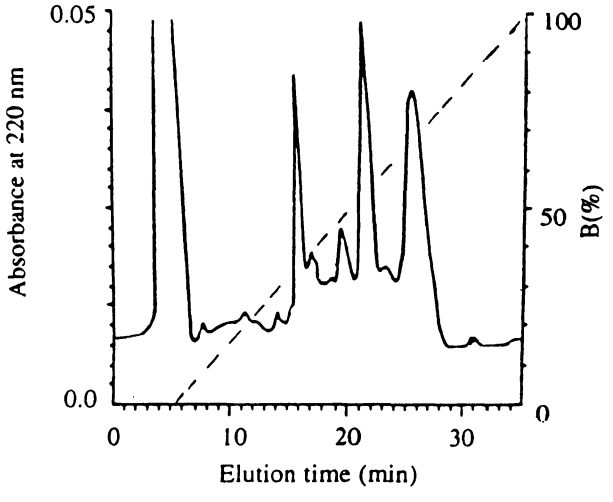


Fig. 83

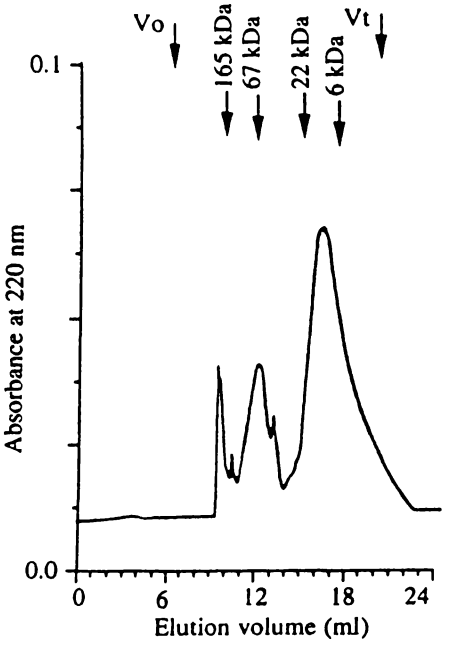


Fig. 84

Fig. 83: Reverse phase chromatography of HMW_{cation} from Fig. 80
Column: ProRPC HR 5/10
Sample: 250 µl of the material in 0.1% trifluoroacetic acid
Eluent A: 0.1% trifluoroacetic acid
Eluent B: 80% acetonitrile in 0.1% trifluoroacetic acid
Gradient: 0-100% B in 30 min
Flow rate: 1 ml/min

Fig. 84: Gel permeation chromatography of MMW_{cation} from the chromatography on Sephadex G25 as shown in Fig. 80.
Column: Superose 12 (analytical grade, HR 10/30).
Sample: 100 µl of the concentrate of MMW_{cation} mixed 1:1 with 0.2 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl pH 7.
Flow rate: 0.5 ml/min.

Isoelectric focussing of the MMW_{cation}

MMW_{cation} showed several bands upon SDS-PAGE. To determine the charges of these components, isoelectric point determination was performed on a PhastSystem using a IEF gel with a pH range of 3 to 9. A 1 µl sample of the concentrate of MMW_{cation} was loaded onto the gel. The results are similar to those shown in Fig. 82. Again the material separated into several bands focussed in the acidic, basic and in the neutral regions. The bands in the basic region were of lesser intensity than the ones in the neutral and the acidic regions.

Reverse phase chromatography of the MMW_{cation}

A 250 µl sample of the concentrate of MMW_{cation} in Fig. 80 prepared in a similar manner as with the sample from HMW_{cation} was subjected to similar chromatography on the reverse phase column. The components resolved into several peaks. This indicates the complexity of these components. There was material that did not bind to the reverse phase column and it appeared in a peak which passed through the reverse phase column in a similar manner to that seen in Fig. 83.

Further study of LMWcation

The component(s) in the third peak in Fig. 80 (LMW_{cation}) had high anti-sperm activity and the apparent molecular weight of this fraction on Sephadex G25 was less than 3 kDa. The material in this peak was subjected to further analysis in attempt to determine its nature and composition.

Gel permeation chromatography under neutral conditions

Fractionation on Sephadex G25 (as in Fig. 80) was conducted to obtain more of the LMW_{cation}. The eluted material (LMW_{cation}) from Sephadex G25 was concentrated to 5 ml (half the original volume of the sample of seminal plasma). A 1 ml sample of this concentrate was freeze-dried and the residue was redissolved in 500 µl of 0.1 mol/l Tris-HCl at pH 7, incubated for 2 h at 37°C then chromatographed on the Superose 12 column and eluted with 0.1 mol/l Tris-HCl at pH 7. The results shown in Fig. 85 indicate that the material eluted at the bed volume with apparent molecular weight of about 1 kDa. The material failed to aggregate. SDS-PAGE indicated a molecular weight of less than 3 kDa (Fig. 80).

Reverse phase chromatography of LMW_{cation}

To check the possibility that the third peak (LMW_{cation}) from Fig. 80 may consist of more than one component with the same molecular weight and eluting at the same place on the column, reverse phase chromatography was conducted. A 500 µl

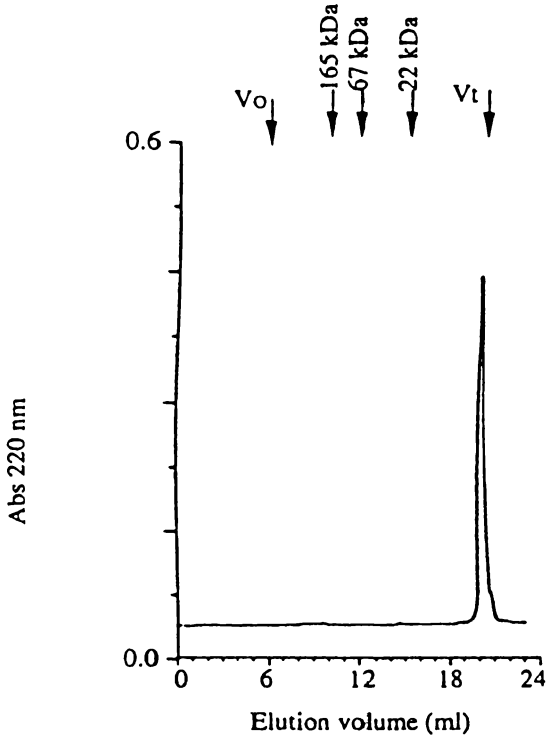


Fig. 85

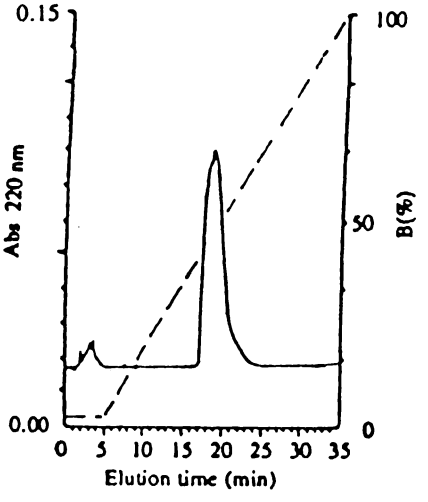


Fig. 86

Fig. 85: Gel permeation chromatography of the LMW_{cation} from Fig. 80.
Column: Superose 12 (analytical grade, HR 10/30).
Sample: 500 µl of the concentrated fraction in 0.1 mol/l Tris-HCl at pH 7.
Eluent: 0.1 mol/l Tris-HCl pH 7.
Flow rate: 0.5 ml/min.

Fig. 86: Reverse phase chromatography of LMW_{cation} from Fig. 80
Column: PepRPC HR 5/5
Sample: 500 µl of the material in 0.1% trifluoroacetic acid
Eluent A: 0.1% trifluoroacetic acid
Eluent B: 0.1% trifluoroacetic acid in 80% acetonitrile
Gradient: 0-100% B in 30 min
Flow rate: 1 ml/min

sample of the concentrate of LMW_{cation} was rotary evaporated to dryness, then redissolved in 0.5 ml of 0.1% trifluoroacetic acid. A 500 µl sample of this was then chromatographed on the PepRPC reverse phase column. As can be seen in Fig. 86 a peak eluted after 13 min from the start of the gradient and one very small peak passed through the column before the start of the gradient. There are obviously no more components eluting along with the LMW_{cation} fraction. The appearance of the very small peak which did not bind to the column could be salt or alternatively may be just an artefact.

Reverse phase chromatography of LMW_{cation} as detailed in Fig. 86 was repeated. Only one peak eluted with the gradient and no other peak appeared. It appeared that this fraction is homogenous and another method was used to check this possibility.

Chromatography on ion exchanger of the LMW_{cation}

Dilution in acetonitrile at pH 1.7 had affected the aggregate. In an attempt to separate the component(s) in the LMW_{cation}, if more than one was present, it was decided to use dilution in acetonitrile at low pH. Because the acetonitrile will prevent binding to the reverse phase column it was decided to use a strong cationic exchanger for this analytical study. Accordingly a 100 µl sample of the concentrate of LMW_{cation} was diluted 100 times with 30% acetonitrile at pH 2, held for 2 h at 4°C then chromatographed on a column of Mono S (a cation exchanger) as detailed in Fig. 87. The separation appeared to be successful and two peaks separated: one passed through the column indicating that the material in this peak was acidic in nature because at pH 2 it was expected that all the positive charges would be exposed and the negative charges would be suppressed. The chromatography was repeated 10 times and identical profiles were obtained and the fractions comprising each peak (fractions 1 to 4 min and 18 to 21 min) were pooled separately for analytical study.

Electrophoresis of the fractions in Fig. 87

The pooled fractions comprising the two peaks in Fig. 87 were desalted on Sephadex G10. The desalted fractions were freeze-dried and then redissolved in 25 µl of 0.1 mol/l Tris-HCl, pH 7. An attempt was made to determine the molecular weight of the components in these solution. Accordingly, a 10 µl sample of each was subjected to SDS-PAGE under reducing conditions on a 20% polyacrylamide gel following the procedure of Giulian *et al.* (1983 and 1985) and silver stained. The results showed a faint band of the cationic fraction (eluted with the gradient) with an estimated molecular weight of 2 kDa, and no band from the fraction that passed through the column unretarded. Attempts were made to improve the appearance of the bands

with no success. Eschenbruch (1980) described a similar phenomenon with regard to the small peptides. However, when the LMW_{cation}, where this material originated from, was subjected to SDS-PAGE on a 20% gel it gave one band at the area with an estimated molecular weight of less than 3 kDa. It is possible that even with modification of the procedure (Giulian *et al.*, 1983 and 1985) it may be that the peptides (considering the low concentration of the fraction) could not stand destaining and diffused out of the gel, a phenomenon common with small peptides.

Gas Chromatography analysis of the unretarded fraction from the chromatography shown in Fig. 87

The component in the unretarded peak from the chromatography shown in Fig. 87 may have been a non-peptide anionic component complexed with the highly positively charged peptide which dissociated from the complex due to high dilution. To check this possibility, gas chromatography analysis of the material in the unretarded peak from Fig. 87 was kindly performed by Jannine Sims (Chemistry Department, University of Waikato). The dried sample was methylated with diazomethane. A 1 ml of prepared diazomethane was added to the dried sample and allowed to react overnight. The methylated sample was then injected onto a gas chromatograph using a HP-1 cross-linked methyl silicon gum column and detection was carried out by Mass Selective Detector. The results indicated the presence of either citrate or succinate. Citrate is present in seminal plasma at a relatively high concentration (Mann, 1964). Shannon *et al.* (1987) observed that citrate forms a strong association with the cationic components in seminal plasma.

5.1.2.4 FURTHER CHARACTERISATION OF THE ISOLATED ANIONIC AND CATIONIC COMPONENTS

Isoelectric focussing

To determine the net charge of the LMW_{cation} in Fig 80, isoelectric focussing was carried out using PhastSystem with a precast mini gel with a pH range of 3 to 9. The samples were: the concentrate of the anionic peptide (WF3) isolated from chromatography on Phenyl Superose and Sephadex G25 (Section 5.1.1.4, Fig. 68) and the concentrate of the cationic fraction (LMW_{cation} from Fig. 80). WF3 was included in this experiment for confirmation of its isoelectric point and purity. A 1 µl sample of each of the concentrates was loaded onto the gel. No bands in the basic area were seen while in the acidic area there was one faint band with estimated pI of 3.5 similar to that found before for WF3 seen in Fig. 74. It was thought that due to the limited pH range (3 to 9) a very basic peptide could have focussed at the edge and

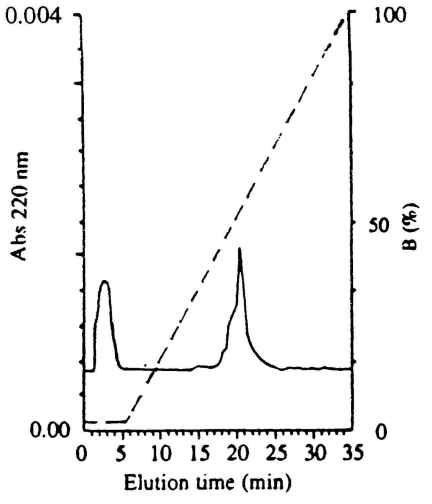


Fig. 87: Chromatography of LMW_{cation} on a cation exchanger.
Column: Mono S HR 5/10.
Sample: 1 ml of the diluted material in 30% acetonitrile at pH 2.
Eluent A: 30% acetonitrile in HCl at pH 2.
Eluent B: 0.1 mol/l NaOH in 2 mol/l NaCl.
Gradient: 0-100% B in 30 min
Flow rate: 1 ml/min.

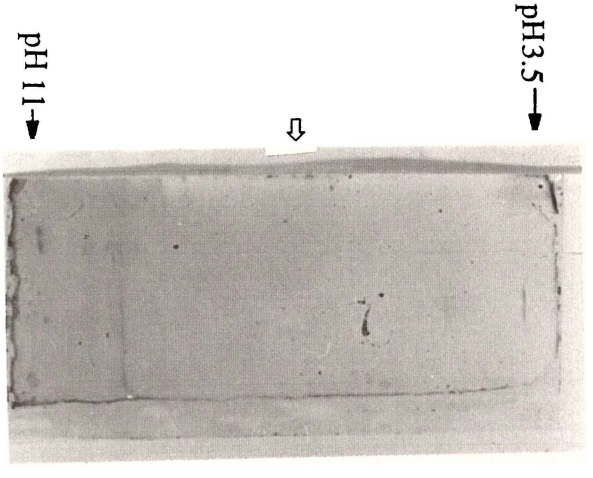


Fig. 88: Isoelectric focussing of the isolated anionic peptide (WF3) and the cationic peptide (LMW_{cation}).
Lane 1: LMW_{cation}. Lane 2: WF3.
⇨ shows point of sample application.

therefore not have been seen. To resolve this, further isoelectric focussing was conducted on the Pharmacia flat-bed apparatus where a higher loading could be made and the pH range extended as described in Chapter 2. Saturated filter papers with each of the concentrate of WF3 and LMW_{cation} were placed on the gel, and the separation was carried out as described in Chapter 2 and the gel was stained with silver. The results in Fig. 88 show two bands at the edges of the gel, one focussed at pH 3.5 and the other focussed at pH 11. The cationic peptide may have a pI higher than 11 especially if it is rich in arginine and lysine.

Amino acid analysis

Amino acid analysis of the cationic and the anionic components (LMW_{cation} from Fig. 80 in Section 3.6.2 and WF3 from Fig. 68 in section 5.1.1.4) was carried out as described in Chapter 2. Samples (100 µl) from LMW_{cation} and WF3 were desalted on Sephadex G10 (XK 16/20 column) eluted with water and freeze-dried. The freeze-dried material was redissolved in 100 µl and 50 µl sample of each was taken for amino acid analysis.

The amino acid composition of the anionic and the cationic fractions are shown in Table 26. The cationic material seems to be rich in lysine and contains arginine which may explain the need for high pH (13) to elute this material from the cation exchanger. The anionic material seems to be rich in acidic amino acids (aspartate and glutamate), however, asparagine and glutamine (neutral amino acids) may have been present in the peptide before hydrolysis and aspartate and glutamate resulted from their deamidation.

Table 26. The amino acid composition of the cationic peptide (LMW_{cation} in Fig. 80) and the anionic peptide (WF3 in Fig. 68).

	mol/100 mol	
Amino acid	**LMW_{cation}**	**WF3**
Aspartate	3.2	14.2
Glutamate	3.0	10.9
Serine	3.2	7.1
Glycine	3.7	8.1
Histidine	1.0	1.5
Threonine	3.2	4.0
Alanine	2.9	6.0
Proline	5.0	8.0
Tyrosine	0.0	6.2
Valine	1.5	6.4
Methionine	0.0	0.0
Cystine+Cystein	0.0	0.0
Isoleucine	2.5	7.3
Leucine	9.3	10.8
Phenylalanine	5.2	9.4
Lysine	51.6	0.0
Arginine	4.6	0.0

Reaggregation study

The isolated anionic peptide from the chromatography on Phenyl Superose and Sephadex (Fig. 68) and the isolated cationic peptide (LMW_{cation}) in Fig. 80 each failed to form aggregates individually. It was thought that the failure to form large aggregates may be due to lack of, or low amounts of one of the components necessary for aggregation. To find out whether a combination of the two components would promote the aggregation process, analytical gel permeation chromatography on Superose 12 under neutral conditions was conducted to study reaggregation and to estimate molecular weights. The labelling of one of the components would show the incorporation of this component into the aggregated molecule. Because the amino acid analysis showed that only the anionic peptide (WF3) contains tyrosine it was chosen for the iodination process. Accordingly, radioiodination of the isolated anionic peptide (300 µg) was carried out to further study reaggregation, by using the Iodogen method of Salacinski *et al.* (1981) as modified by Hodgkinson *et al.* (1987) as described in the Chapter 2.

Mixtures of ^{125}I-labelled WF3 (anionic peptide) and LMW_{cation} (cationic peptide) each containing 22 µg protein/100 µl 0.1 mol/l Tris-HCl buffer, pH 7 were prepared in the following proportions: 50:50, 60:40 and 30:70 respectively and incubated for 2

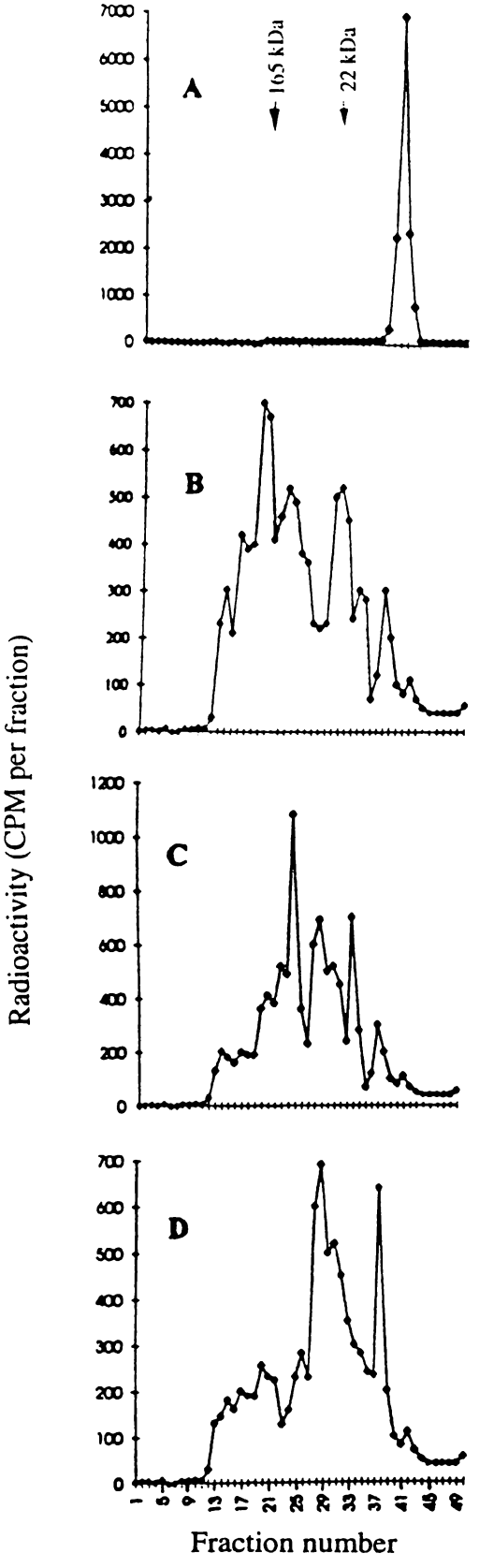


Fig. 89: Gel permeation chromatography of:
a) ^{125}I-WF3,
b) mixture 60:40% of ^{125}I-WF3 and LMW_{cation} respectively,
c) mixture 50:50% of ^{125}I-WF3 and LMW_{cation} respectively,
d) mixture 30:70% of ^{125}I-WF3 and LMW_{cation} respectively
Column: Superose 12 (analytical grade, HR 10/30).
Sample: 100 µl of a, b, c or d at pH 7.
Eluent: 0.1 mol/l Tris-HCl pH 7.
Fraction volume: 0.5 ml.
Flow rate: 0.5 ml/min.

h at 37°C. After incubation, the samples (made up to 0.5 ml with 0.1 mol/l Tris-HCl, pH 7) were chromatographed on the analytical column of Superose 12 eluted with 0.1 mol/l Tris HCl, pH 7. Fractions (0.5 ml) were collected and the content of ^{125}I in the fraction was measured in a gamma counter. The results shown in Fig. 89 indicate that the anionic peptide eluted at the bed volume of the Superose 12 column when chromatographed alone, but after incubation with the cationic peptide in various proportions the two components formed an aggregate and full aggregation was seen when the proportion was 60:40 of the anionic and the cationic peptides respectively.

Test for anti-sperm activity

The isolated cationic peptide (LMW_{cation}) was found to have high anti-sperm activity. In contrast the isolated anionic peptide of similar molecular weight did not reduce the motility of sperm. To determine what effect a combination of the two components had on the sperm motility, a sample of the four-fold concentrate (quarter of the original volume of seminal plasma) of the cationic (Section 5.1.2.3) and the anionic (Section 5.1.1.2) peptide were mixed 40:60 (v/v) and incubated for 2 h at 37°C. The mixture was then mixed 1:1 with DS14G to be tested for anti-sperm activity. A sample from the concentrate (half of the volume of the original seminal plasma) of LMW_{cation} was mixed 1:1 with DS14G. Aliquots of 100 µl of the prepared samples were made up to 1 ml final volume with sperm suspension in 14G to give 10% (v/v) concentration of the fraction and 10-12 x 10^6 sperm/ml. The motility of sperm was assessed immediately after addition and after 18 h incubation at ambient temperature (18 to 21°C). The results are shown in Table 27.

Table 27. Effect of the mixture of LMW_{cation} and WF3 on the motility of washed bull sperm.

The values for motility are the percentage of motile sperm immediately after addition and after 18 hours incubation at ambient temperature. Values are mean ± s.e.m (n=3). All fractions were at 10% of their original concentration in seminal plasma.

Treatment	Protein conc. (mg/ml)	% Motile	
		0 h	18 h
Control (diluent)		83.3±1.7[a]	73.3±1.7[a]
LMW_{cation}	0.11	73.3±1.7[b]	40.0±2.9[d]
Mixture of LMW_{cation} and WF3	0.33	80.0±4.5[a]	56.7.±6.1[c]
Seminal plasma*	4.30	83.3±1.7[a]	68.3±1.7[b]

**Seminal plasma at 10% concentration for comparison.*

Like superscripts within a column indicate no significant difference between means ($p<0.05$).

The LMW_{cation} significantly reduced sperm motility immediately and after 18 h incubation. On the other hand the combination of the active (cationic) and the inactive (anionic) peptides reduced the activity of the cationic fraction.

5.1.3 SUMMARY AND CONCLUSION:

Prolonged exposure to acetonitrile under acidic conditions releases the low molecular weight components from the macromolecular aggregate. These low molecular weight components are detrimental to sperm motility. The material that passed through the ion exchanger appeared to be an aggregate which under disaggregating conditions further separated into several fractions with varying degrees of anti-sperm activity (Section 5.1.1, Table 21). Immobilisation on Phenyl Superose resulted in fractions that possess high anti-sperm activity (WF2), but also fractions which did not exhibit any anti-sperm activity (WF3). The fraction with no anti-sperm activity appeared to be a pure anionic peptide.

The material that bound firmly to the cation exchanger was then dissociated from the column with NaOH. This fraction under disaggregation conditions separated into three fractions. The HMW_{cation} and MMW_{cation} were still aggregates but the LMW_{cation} appeared to be a single peptide with molecular weight less than 2 kDa and a pI of 11. It was rich in lysine and arginine. Neither the anionic nor the cationic peptide formed aggregates on its own, but a combination of both peptides succeeded in forming aggregates of higher molecular weight.

The cationic peptide exhibited high anti-sperm activity, while the anionic did not show any effect on sperm. A combination of both attenuated the anti-sperm activity of the cationic peptide.

5.2 DISCUSSION

Most of the protein in bovine seminal plasma is in the form of aggregates of low molecular weight components held together by ionic and hydrophobic interactions. Immobilisation of these aggregates on a cation exchanger and prolonged exposure to acetonitrile under acidic conditions releases the low molecular weight components from the macromolecular aggregate due to the break-down of these forces. The cationic material of low molecular weight is left attached to the ion exchanger while the anionic components are washed off.

It was assumed that the material eluted with acetonitrile from the cation exchanger had a higher proportion of the acidic peptides (hydrophobic in nature) than the basic peptides (hydrophilic). Similarly, it was assumed that the material eluted with NaOH contained a higher proportion of basic peptide (hydrophilic) than anionic peptides (hydrophobic).

Disaggregation of the high molecular weight aggregates was evident when the predominantly anionic material eluted with the acetonitrile from the cation exchanger was further processed on a Sephadex column under disaggregating conditions (Fig. 63). The appearance of peaks of estimated molecular weight less than 5 kDa is an indication of the disaggregation process. Although separated into four fractions, it does not appear that the anti-sperm activity itself had partitioned into any one specific fraction. The separated components were small aggregates that retained anti-sperm activity. They appeared to be of a low molecular weight under disaggregating conditions due to the break-down of the hydrophobic and ionic interactions, however, under neutral conditions they reaggregated to a higher molecular weight.

The material eluted with acetonitrile from the cation exchanger was then immobilised on a Phenyl Superose column. The rational for this was that the anti-sperm activity could possibly be partitioned into a hydrophilic or hydrophobic component. Because the material eluted with acetonitrile is predominantly acidic, it was thought to be a good source for the isolation of the anionic peptide. However, this separation did not appear to have dissociated the anti-sperm activity since the anti-sperm activity was present in both hydrophilic and hydrophobic components.

The hydrophilic components were all in an aggregated form with low anti-sperm activity even though they originated from unbound material from the cation exchanger which exhibited high anti-sperm activity (Table 22). The process of disaggregation may have caused a redistribution of the anti-sperm activity component(s) and altered their ability to act on sperm. The hydrophobic components from Phenyl Superose retained high anti-sperm activity although WF3, a pure anionic hydrophobic peptide was inactive. This leads to the suggestion that by disaggregation, the anti-sperm activity is redistributed preferentially in the hydrophobic aggregates.

It was thought that the anti-sperm activity is due to substances cationic in nature and the observation that the apparently anionic hydrophobic fractions (WF1 and WF2) were very active against sperm ran against this hypothesis. However, on isoelectric focussing of these fractions (WF1 and WF2), there was a fair amount of cationic

material observed. It is reasonable to speculate that these anionic proteins might have a cationic patch on the molecule similar to the anionic proteins described by Manjunath and Sairam (1987). A further discussion on this is presented in Chapter 6.

On the contrary, the free cationic peptide (LMW_{cation}) was very active on sperm although it was not an aggregate as determined by SDS-PAGE and chromatography. The cationic aggregates (MMW_{cation} and HMW_{cation}) were both active on sperm (Table 25) and the activity in comparison to the anionic hydrophobic material was much higher. This reiterates the point that it is the cationic material that is responsible for the anti-sperm activity. Therefore, when seminal plasma is fractionated into anionic and cationic components, the anti-sperm activity is primarily restricted to the aggregates in the anionic material and absent in the free anionic peptide. This is not the case with the cationic material as the anti-sperm activity was distributed to varying degrees in both the aggregates and the free cationic peptide. Combining the free anionic and free cationic peptide reduced the anti-sperm activity of the cationic peptide suggesting a role for the anionic material in counteracting the anti-sperm effects by aggregation. It is possible that the relative proportion of the anionic and the cationic material in the aggregate determines the level of anti-sperm activity. This could explain the relative anti-sperm activities of WF1 and WF2. Both are anionic aggregates with possibly different proportions of cationic material.

Baas (1985) reported that a cationic fraction from bovine seminal plasma was more detrimental to sperm than the anionic fraction. It is possible that both these fractions are aggregates and their relative activities are due to the different proportion of the anionic and the cationic peptide contained within.

Strongly anionic compounds such as amaranth and DNA provide protection to sperm against the detrimental effect of bovine seminal plasma during cryopreservation (Shannon and Curson, 1983). Evidence suggests that amaranth protects sperm through its binding to the damaging cationic component(s) in seminal plasma (Shannon and Curson, 1983)

An important aspect that has surfaced in this study is the nature of the aggregate and that even under fairly harsh conditions only partial disaggregation occurred. The reason for this could be that the cationic peptide is strongly associated in the aggregate. To detach the cationic material from the cation exchanger required high pH. The cationic peptide contains arginine and lysine which are highly basic amino acids. The electrophoretic analysis showed that both HMW_{cation} and MMW_{cation}

aggregates contained some anionic material. It is proposed that the cationic aggregate is arranged in such a way that there is a preponderance of positive charges on the surface which promote attachment to the cation exchanger and the anionic material is buried within the aggregate therefore preventing it from being washed off with acetonitrile.

In this Chapter a procedure has been developed that is successful in separating a free cationic (LMW_{cation}) and a free anionic (WF3) peptide in a homogenous state. The purity of these two peptides was confirmed by several methods. The anionic peptide was rich in aspartate and glutamate (but these could have been asparagine and glutamine before hydrolysis) and the cationic peptide was rich in lysine and arginine.

Attempts to further characterise LMW_{cation} on a cation exchanger (Mono S) using high dilution, acetonitrile and acid resulted in anionic material dissociating from the molecule. On later analysis, this was found to be citrate or succinate, both of which are strongly anionic which explains why it bound firmly to the cationic peptide. A similar observation that cationic proteins from bovine seminal plasma are firmly associated with citrate has been recorded by Shannon *et al.* (1987) and San Agustin and Lardy (1990).

Both the anionic and cationic peptides failed to reaggregate on their own. However, on combining the two they formed aggregates of different molecular weights depending on the proportion of the individual peptides. This may explain why the anionic hydrophobic and hydrophilic material from Phenyl Superose chromatography did not reaggregate to a substantial degree while the cationic material formed aggregates similar to that seen in seminal plasma. A further discussion of this follows in Chapter 6.

The most convincing evidence to date on the process of disaggregation/aggregation in bovine seminal plasma has been presented by Shannon *et al.* (1987) and Hameed *et al.* (1991). A similar system has been reported by Acott and Hoskins (1978) when they described the nature of a forward motility protein from bovine seminal plasma. Under neutral conditions, the activity was seen in all fractions ranging from 10 to 1 600 kDa. On dissociation with urea or SDS the activity shifted to the lower molecular weight levels of 10 to 25 kDa. When the dissociating conditions were removed the low molecular weight components reaggregated to form the profile seen before dissociation.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

In the investigation described in this thesis, an attempt was made to explore the anti-sperm activity in bovine seminal plasma. This activity was demonstrated to be spread over a wide range of molecular weight components (Shannon *et al.* 1974). It was presumed that the anti-sperm and antibacterial activity in seminal plasma were due to the same components. The proteins in seminal plasma exist in a mass of aggregates. The anti-sperm and the antibacterial activities reside in this mass without a clear identity. The present study clearly identified the anti-sperm component. Although the anti-sperm and the antibacterial activity were made up of the same components, they differ fundamentally.

Preliminary observations in Chapter 3 demonstrated that, under neutral conditions, anti-sperm activity is prominent in the low molecular weight fractions while the antibacterial activity is more pronounced in the high molecular weight fractions. Dissociation of seminal plasma macromolecular aggregates increased the relative anti-sperm activity but not the antibacterial activity. Small antibacterial peptides have been isolated from seminal plasma (2.2 kDa by Eschenbruch, 1980; 3.5 kDa by Hameed, 1987) but were lower in activity in comparison with the antibacterial activity seen in the high molecular weight fractions. Unfractionated seminal plasma undoubtedly had the highest antibacterial activity.

A preliminary investigation showed that for maximal antibacterial activity, the components need to be in an aggregated state while the anti-sperm activity is predominant in the lower molecular weight components. A hypothesis for this has been presented in Chapter 3.

It can be speculated that the buried anti-sperm activity in the aggregate is like a "time-bomb" and the aggregating factors are the safety pin. The release of the low molecular weight components is probably not a sudden burst but rather a slow retarded release. This process was shown by dialysis of seminal plasma under dissociating conditions (acid and citrate) where it appears to be released slowly since it was found in the diffusate even after several changes of the diffusate under these conditions. Interfering with ionic interaction using citrate as a disaggregating agent, released low molecular weight substances, yet further dissociation of the high molecular weight fractions on Sephadex G25 under acidic conditions generated highly

active low molecular weight fractions. This clearly suggests the high anti-sperm activity of the low molecular weight components is masked by the high molecular weight aggregates and that aggregation plays a role in minimising the damaging effects on sperm.

The low molecular weight fractions contain both anionic and cationic components. This is evident from the results of the isoelectric focussing presented in this thesis. Although low molecular weight fractions may seem to be of similar molecular weight, they are considerably different in their anti-sperm activity. The difference in the content of the cationic and the anionic components seen from the results of the isoelectric focussing of the fractions resulting from the dissociation of HMWcitr on a Sephadex column is an example of this (Fig. 22). These differences in the composition may have contributed to the variation in the anti-sperm activity within the fractions. It is tempting to speculate that the unique combination of charge and shape of the molecules determines their relative activities.

In Chapter 3, anti-sperm activity was shown to be due to the low molecular weight component(s). However, conclusive evidence of the identity of the components responsible for the anti-sperm activity could not be drawn at that stage. This is because the low molecular weight components behaved differently during gel permeation chromatography in various aqueous media of different pH values (pH 1.7 and pH 7). While these components eluted at a position corresponding to a molecular weight of less than 3 kDa at pH 1.7, they eluted as aggregated molecules of molecular weight in the range 5 to 150 kDa at pH 7.

The macromolecular aggregate in seminal plasma is presumed to be held together by forces such as hydrophobic attraction and strong ionic interaction. To isolate the active molecule an attempt was made in Chapter 4 to dislodge these forces. The primary reason for this was to explore the hypothesis that the activity buried within the aggregate can be accessed by weakening these forces. Isopropanol precipitation at low pH was used as the main technique to achieve this. With the "safety pin" now removed or weakened, Phenyl Superose, a mild hydrophobic interaction medium was used to successfully isolate the free anionic peptide in homogenous form along with other fractions which remained in aggregated form.

The major protein in bovine seminal plasma is reported to be anionic in nature with a molecular weight estimated to be between 15 and 16 kDa (Kemme *et al.*, 1986). Later, Aumüller *et al.* (1988) proposed that this protein may be involved in the onset

of hyper-activation of sperm motility. In a recent report, Kononov *et al.* (1991) reported that all but one of the anionic fractions they isolated from bovine seminal plasma decreased the duration of motility of washed bull sperm. It has also been reported that an anionic peptide with a molecular weight of 5.7 kDa from boar seminal plasma inhibits sperm motility and possesses lytic activity against *Micrococcus lysodeikticus* (Strzezek *et al.*, 1992).

The isolated pure anionic peptide with estimated molecular weight of 2 kDa did not reduce the motility of sperm after 18 h incubation. This was interesting because all of the other fractions that remained in an aggregated state retained anti-sperm activity to varying degrees. This observation suggests the possible presence of an active anti-sperm component in the aggregates. The variation in activity amongst the fractions may be due to having the inactive and the active component in various proportions. Peptides with no antibacterial activity from seminal plasma have been reported by Hameed (1987) with molecular weights between 2.3 and 3 kDa. These peptides also failed to reaggregate to higher molecular weight.

Anti-sperm activity in bovine seminal plasma has been reported to be associated with the cationic polypeptides (Baas, 1985), however, no detailed characterisation of this material was attempted. The present study fully investigated the cationic components in seminal plasma. The observations from the work in Chapter 3 and 4 clearly indicates that interfering with the ionic and the hydrophobic attraction are useful tools in disaggregating the macromolecule. In Chapter 5 this study was extended. The reason for using a cation exchanger was to keep the cationic material separated from the other component(s) in the aggregate. Whole seminal plasma was used as starting material to obtain the maximum amount of the cationic material from the aggregate. Extended exposure to acetonitrile in low pH succeeded in separating a cationic peptide with estimated molecular weight of 1.5-3 kDa. However, material that washed out of the cation exchanger and the material bound to the cation exchanger were still in aggregated form. Only upon further chromatography under disaggregative conditions using acetonitrile at pH 1.7, was a small amount of the cationic peptide released. It is worth mentioning that all the other fractions obtained from the cation exchanger were a complex of molecules varying in their molecular weights and charges. Designation such as HMW_{cation} was not entirely a true representation of this fraction since isoelectric focussing revealed the presence of bands in both regions: the cationic and the anionic. The presence of seminalplasmin in bovine seminal plasma, a basic protein with a molecular weight of 5.4 kDa, is well documented (Reddy and Bhargava, 1979; Theil and Scheit, 1983, Shivaji, 1984;

Shivaji and Bhargava, 1987, Scheit *et al.*, 1985). The HMW_{cation} fraction would contain this protein and some other proteins and peptides which explains its detrimental effect on sperm motility. The early reports from Hibbitt and Benians (1971) that seminal plasma contains basic proteins which can damage the integrity of the sperm membrane are in agreement with the present study.

The purified cationic peptide failed to reaggregate by itself, but unlike the isolated anionic peptide, exhibited high anti-sperm activity. Anti-sperm activity varied amongst all the fractions from the cation exchanger probably for the same reasons given above. These cationic fractions may exist in forms ranging from the monomer to a large molecular weight polymer. Alternatively, the cationic peptide may be attached to a variety of different proteins in seminal plasma (Shannon *et al.*, 1975). The latter reason could explain the appearance of some aggregates which were less active against sperm.

An attempt was made to further analyse the cationic peptide (LMW_{cation}). Dilution with acetonitrile at low pH and then separation on Mono S showed that components in this basic peptide separated into two peaks, one of which was unretarded. This separation could not be detected on the Superose 12 column as the two components with possibly similar molecular weights eluted at the same place. The existence of a monomeric anti-sperm peptide (less than the smallest aggregate of 3.5 kDa), was doubted. The monomeric peptides could be speculated to be a very stable aggregate of two components held strongly together. However, further analysis of the unretarded peak showed that it composed of citrate or succinate. This unequivocally proved that the cationic peptide was a monomer.

Conformational changes in proteins may occur during processing of seminal plasma. A good example is provided by Rufo *et al.* (1982) with regard to the transformation of seminalplasmin (caltrin), a protein of molecular weight of 5.4 kDa. The speculated role of seminalplasmin (caltrin) is the prevention of premature calcium uptake by freshly ejaculated sperm. Seminal plasma may play a role in maintaining seminalplasmin (caltrin) in a conformation that is inhibitory to this influx. The transformation to an enhancer of calcium uptake would then be due to a conformational change in seminalplasmin (caltrin) induced by an appropriate medium. Rufo *et al.* (1982) found that freshly isolated caltrin initially behaves as an inhibitor of calcium uptake in bovine sperm. It subsequently undergoes a remarkable transformation with time to become an enhancer of calcium uptake. Recently, San Agustin and Lardy (1990) reported that during the extraction of caltrin, removal of

non-peptide anionic material reversed the caltrin activity from enhancer to inhibitor of calcium uptake.

As the system in seminal plasma is very complex due to the existence of the aggregation system, the damaging activity of fractions separated from seminal plasma may increase or decrease due to conformational changes. Breaking down the aggregates may expose more of the active region and provide more access for the activity to be exerted in a more dramatic way than when it is masked by the aggregate. In the present study, the activity of an active fraction from the cation exchanger (*i.e.* the LMW_{cation}) was reduced when incubated with the inactive anionic peptide obtained from the Phenyl Superose column (*i.e.* WF3). It seems that the activity is reduced due to aggregation and formation of a new complexes, which was seen on chromatography after incubation of both components together.

A GENERAL THEORY ON THE AGGREGATION / DISAGGREGATION OF SEMINAL PLASMA PROTEINS WITH REFERENCE TO THE RESULTS IN THIS THESIS.

Seminal plasma is rich in proteins and it is the physiological medium for sperm when they are ejaculated. Unfractionated seminal plasma does not possess the same level of anti-sperm activity as seen with the cationic peptide as mentioned above. The sperm survive in this medium for extended periods of time with a progressive loss in motility and viability.

The process of aggregation in seminal plasma and the components that contribute to this process such as the anionic and the cationic peptides is the subject of discussion. It is presumed that both these components interact with each other in a certain ratio to reach a stable aggregate with minimum sperm damaging activity. In their bound state the anionic components do not seem to completely neutralise the effects of the cationic components against sperm, but they form a whole series of aggregates with different levels of activity against sperm. This theory is supported by evidence on gel permeation chromatography of the combination of the anionic and cationic peptides and the reduced activity of the cationic peptide after combining with the anionic peptide as described in Chapter 5.

It is interesting that there are a number of reports that describe a "major protein" in seminal plasma, and all ascribe molecular weights in the range of 14 to 20 kDa. Kemme *et al.* (1986) described a protein of molecular weight 15 to 16 kDa.

Collingwood (1990) mentions a sperm damaging protein of 16 kDa, Hameed (1987) and Eschenbruch (1980) reported that an aggregate of 500 kDa disaggregated to a 20 kDa aggregate with antibacterial activity. Another antibacterial component from seminal plasma with estimated molecular weight of 15 kDa has been reported by Schollum (1977). Many of these isolated proteins have a great deal of similarity in amino acid composition and net charge, yet they have been assigned different names and functions. Also, acidic proteins isolated by Esch *et al.* (1983), Kemme *et al.* (1986), Manjunath and Sairam (1987) and Chandonnet *et al.* (19990) have a similar charge to each other and are in a molecular weight range of 14 to 20 kDa with almost identical amino acid composition. This leads to the speculation that a stable aggregate in seminal plasma exists in the molecular weight range of 14 to 20 kDa. In a similar vein, cationic proteins with fairly similar molecular weights have also been reported (Scheit, 1986).

The reported small differences in the amino acid composition or molecular weight of these proteins mentioned above could be due to the slight differences in the ratios of the two peptides that contribute to the formation of the aggregate. Evidence for this was obtained in this study when the extent of aggregation varied depending on the ratio of the two peptides present (radiolabel study, Chapter 5). This in turn could also explain the differing biological activities of these proteins. It is erroneous to suggest that some of these isolated proteins are homogenous: in fact they could very well be stable aggregates with minor differences. If these proteins were to be subjected to similar disaggregation studies it could be proved that the primary building blocks are the cationic and anionic peptides as shown in the present study. Hameed (1987) has reported that the 20 kDa aggregate can be completely disaggregated to components of approximately 1 kDa.

The stable aggregate was demonstrated to be strongly held together. The technique used in the present study involving cation exchanger with acetonitrile in acid, hydrophobic interaction column and dilution with acetonitrile in acid was necessary to break down the macromolecular aggregate. It would be interesting to subject the proteins of molecular weight 14 to 20 kDa described by other workers or the ones with high molecular weight to the same disaggregation technique used in the present study.

It was noticed in the present study that components did not disaggregate on the column of Superose 12 with acetonitrile unless acidic conditions were present. The experiment has been conducted both with and without the acid (results not reported).

High molecular weight peaks were still obtained when the chromatography was carried out with 30% acetonitrile in the absence of acid indicating the importance of ionic interactions. There is the possibility that dilution in the absence of acid and acetonitrile could cause disaggregation. This has not been explored in this study.

Concentration also appeared to be an important factor in stabilising the protein aggregates. Dilution of samples in acetonitrile at pH 1.7 was necessary for complete disaggregation of the large aggregate. The failure of the resulting disaggregated material to resume a high level of aggregation may be due to a low concentration of one or both of the components, (*i.e.* either the cationic or the anionic peptide present in insufficient concentration). The disaggregation of the diluted sample probably occurred as a result of low concentrations of the sample which decreased the probability of interaction between the molecules involved in aggregation, thus complete disaggregation resulted.

The formation of an aggregate required that the two components be present in a certain proportion and sufficient concentration. Once the two components are introduced to one another reaggregation could occur at neutral pH. In the present study, it was demonstrated, by radiolabelling the anionic peptide , that reaggregation could occur to high molecular weight. Radioactivity was detected in the various peaks indicating the incorporation of the anionic peptide in the aggregates.

It is suggested that a critical proportion of the two peptides is required for reaggregation to take place, and below this proportion the components fail to reaggregate, despite both the anionic and the cationic component being present. The finding by Hameed (1987) that some peptides from seminal plasma of 2-3 kDa failed to reaggregate is in general agreement with the findings in the present study.

The reason a diversity of aggregates was found could be that the two peptides (the cationic and anionic) were assembled in different ratios, or that the concentrations of the samples could have affected reaggregation. This is because a concentrated sample would physically facilitate aggregation, whereas a diluted sample would not. Hameed (1987) made similar observations when studying reaggregation.

It could be suggested that the presence of low molecular weight components could be due to proteolysis of the large aggregates in seminal plasma. However, there is ample evidence provided in the present study on the process of reaggregation when the disaggregative conditions are removed. There is some proteolytic effect in

seminal plasma but the extensive disaggregation seen in the presence of acid, citrate, acetonitrile and dilution indicates that it is due to the breakdown of the ionic and hydrophobic forces that hold the aggregate together rather than proteolysis.

De Lamirande *et al.* (1986) reported that the macromolecules in seminal plasma do not have intrinsic motility inhibition after the removal of a low molecular weight motility inhibitor by dialysis under acidic conditions. The present study is not in agreement with the existence of a large inactive molecule that works as a carrier. This is because when the high molecular weight components in the retentate of dialysed seminal plasma were subjected to disaggregation, they generate low molecular weight components with high anti-sperm activity. However, the existence of a high molecular weight component with low anti-sperm activity could be explained by the aggregating system. Nature may have evolved this system as a protection to sperm and the active low molecular weight components may have its active sites buried when associated in a large molecular weight. The release of this low molecular weight component would expose the active sites and increase the damaging activity. Another possible explanation is that the target site on the sperm membrane can be fitted ultimately only when the molecule is small enough to fit in that site.

A HYPOTHESIS FOR THE MODE OF ACTION OF SEMINAL PLASMA DAMAGING FRACTIONS ON SPERM MEMBRANE

The mode of action of the damaging component(s) on sperm is not sufficiently clear. There is evidence that cationic proteins bind to sperm membranes (Shannon and Curson, 1983) and may exert their damaging activity in a manner similar to that described for bacterial membranes (Farley *et al.*, 1987, Rosenthal and Buchanan, 1974). It is proposed that the fractions in aggregated form retain some of the damaging activity due to the presence of cationic charges on the molecules. The latter could act as the cationic head (*i.e.* the protein will act in a detergent-like manner with a hydrophobic body and cationic head). It was suggested that the anionic sperm-damaging protein may act by removing a stabilising cationic species from the plasma membrane, resulting in its destabilisation and loss of integrity (Collingwood, 1990). The conclusion of Collingwood (1990) was based on the assumption that the protein tested was anionic, however, there was no report on characterisation of charges or amino acid analysis. This protein has been shown to be an aggregate and it is possible that it may have a cluster of positive charges in the molecule. This may explain the role of this protein in binding to the negatively charged groups on sperm membrane and thus exerting its damaging activity. Miller *et al.* (1990) reported that anionic

proteins with similar molecular weight (15-17 kDa) peripherally adsorbed onto the sperm membrane and could be removed by a hypertonic medium. This loose binding indicates that it is mainly held by ionic interaction. This binding also possibly can be through a cationic cluster.

It was established that the damaging activity of seminal plasma on sensitive bacteria results from damage to the plasma membrane (Jarvis, 1979). Shannon *et al.* (1974) observed that the addition of lecithin, a membrane component, to suspensions of sperm or bacteria containing bull seminal plasma had a protective effect and reduced the damaging effect of the seminal plasma. The damaging effect of these components in seminal plasma may be due to a loose association with the sperm membrane. This damaging effect was substantially reduced by the addition of egg yolk (Shannon and Curson, 1983).

In the present study, WF3, a pure anionic peptide had no effect on sperm motility. However, its role in regulating sperm motility cannot be ruled out. It is possible that the anionic material in seminal plasma counteracts or reduces the effect of the active cationic material on sperm and thus plays a part in the protection of the sperm. An acidic protein from bovine seminal plasma, anti-seminalplasmin, reverses the effect of seminalplasmin, a cationic protein in bovine seminal plasma, on sperm motility and fertility (Rao and Bhargava, 1985). This system may have existed to regulate fertility, complexing in macromolecular form and reducing/delaying the anti-sperm activity.

De Lamirande *et al.* (1986) suggest that motility inhibition due to a cationic motility inhibitor is due to its action on the sperm axoneme and inhibits the dynein ATP-ase activity. Iwamoto *et al.* (1992) purified a cationic protein from boar seminal plasma that inhibits sperm motility and dynein ATPase. The cationic fractions in the present study appeared to initially hyperactivate the sperm (*i.e.* progressive and wriggling motion) followed by severe head-to-head agglutination of sperm. It possible that the low molecular weight fractions interfere with motility by mechanism that enable them to be transported by the plasmalemma to the axoneme.

As yet a universal mechanism for damaging activity has not been established. The role of charge and hydrophobic interaction between cationic peptides or proteins and sperm or bacteria is not fully understood. However, for some cationic proteins, the role has been established. Weiss and Elsbach (1983) provided evidence suggesting that the action of purified cationic bactericidal/permeability-increasing protein from

neutrophils, on susceptible Gram negative bacteria requires saturation binding to negatively charged surface sites. This must be followed by hydrophobic interaction with the outer membrane in order to produce its antibacterial effects. Thus, post-binding events irreversibly trigger the bactericidal action of the cationic protein.

By the use of radiolabelled antimicrobial cationic protein isolated from neutrophil granules Farley *et al.* (1988) demonstrated that a direct correlation exists between protein binding to the microbial surface and its antimicrobial effect.

The presence of the hydrophilic cationic and the hydrophobic anionic peptides is required to fulfil the requirements mentioned above for the antibacterial activity to be exhibited. Maximal antibacterial activity was recovered after combining both peptides (P.C Molan, personal communication). However, the anti-sperm activity was fulfilled by the active hydrophilic cationic peptide alone and this activity was reduced when combined with the anionic peptide. This is in agreement with the theory proposed in Chapter 3 which suggests that the anti-sperm and the antibacterial activities are different.

It is logical to presume that the size of the molecule dictates its respective activity such as antibacterial or anti-sperm activity. For instance the antibacterial action would require the large aggregate to locate on a specific site on the bacterial membrane, however, the anti-sperm molecule is relatively small and probably locates on a totally different site on the sperm membrane. An alternative mode of action for the anti-sperm peptide could be that small peptides may be capable of entering the membrane of sperm via peptide permeases, if they satisfy the structural requirements of peptide permeases, and thus attack an intracellular target. When this anti-sperm molecule is in the form of an aggregate, its activity is lessened. Therefore, the lack of activity of some aggregates in the present study may be the result of inappropriate size, conformation and/or configuration of the aggregate.

It also possible that monomers cannot exert anti-sperm activity unless their sites on the surface of the sperm are stabilised by cooperation with a lipopolysaccharide component from the membrane or a hydrophobic domain on the monomer molecule itself. In the case of the active cationic peptide, amino acids analysis revealed the presence of basic and hydrophobic amino acids on its molecule.

THOUGHTS FOR THE FUTURE

The continuous aggregation/disaggregation system observed in seminal plasma seems to be unique and this may explain the discrepancies seen in the description of molecular weight of some proteins in other species. These protein(s) or peptide(s) could be aggregates of small molecular weight peptides. Until recently very little was known about the aggregation/disaggregation systems of these protein(s) or peptide(s). It would be interesting to examine their behaviour if they were subjected to a disaggregation study similar to the one used in the present study.

The way the different aggregates in seminal plasma exert their anti-sperm activity and their interaction with the sperm surface should be studied in more detail. The physico-chemical nature of the interaction could explain the specificity of some of these anti-sperm protein(s) or peptide(s) against sperm, and if there is specificity, what contribution the aggregates may offer in regulating sperm survival and fertility. Further work should be undertaken for understanding the significance of the anti-sperm peptide which arose from the disaggregation of the large molecular weight aggregate, the way it functions in the native state and its possible biological effect if it is one of the various biologically active proteins reported in the literature. More study on the inactive peptides may reveal a way of counteracting the damaging effect of active component(s) in seminal plasma. This may contribute to a better preservation process and reduction in the number of dead sperm.

If physiological conditions were to cause transformation in the shape and size of the aggregates, this could lead one to accept the existence of a parent aggregate which under certain conditions would disaggregate to the correct size to exert its biological role. Alternately, different sizes and shapes could already exist. Further study on these points is necessary.

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