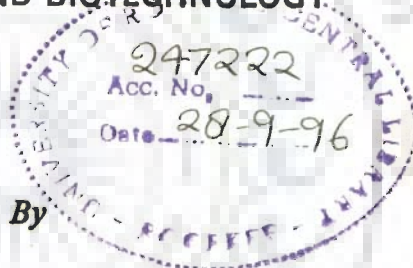# BIOCHEMICAL INVESTIGATION ON THE MATURATION OF SPERM IN EPIDIDYMIS

A THESIS

submitted in fulfilment of the
requirements for the award of the degree
of
DOCTOR OF PHILOSOPHY
in
BIOSCIENCES AND BIOTECHNOLOGY

By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled BIOCHEMICAL INVESTIGATION ON THE MATURATION OF SPERM IN EPIDIDYMIS in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biosciences and Biotechnology of the University is an authentic record of my own work carried out during a period from January 1989 to July 1994 under the supervision of Dr. B.M.J. Pereira.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

(NEETI SRIVASTAVA)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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ABSTRACT

The sperm produced in the testis are initially non functional, lacking the ability for forward progressive motility and fertilizing capacity. But they acquire these traits after spending considerable periods of time in the epididymis. The epididymis nurtures, nourishes and provides the right ingredients that transform the sperm in a way it can accomplish its task of successful fertilization. This transformation is reflected in the form of morphological, biochemical and physiological changes in sperm. The sperm surface too undergoes remarkable remodelling during epididymal transit. The current study tries to identify some of the sperm surface changes and understand the mechanism(s) by which they are brought about.

To accomplish this, goat epididymal sperm has been used as model for two main reasons. First, the goat epididymis is large sized and therefore sperm can be recovered from multiple sites of the duct. Second, the study material is easily available since goats are routinely slaughtered at the local abattoir for meat consumption.

So far, most investigators have studied the epididymal tissue, luminal fluid and sperm as a single unit making it difficult to interpret data. The lack of appropriate methods for the separation of these three entities has been solely the reason for such an approach. But over the years techniques such as micropuncture and retrograde flushing have become available. But these methods have limited practical utility when more segments of the epididymis are involved. In the present study a novel approach has been used to

separate tissue, fluid and sperm. Essentially the method involves subjecting 1mm slices of epididymis in desired buffer to an evacuation pressure of 200mm Hg for 2 minutes and then filtering the suspension through cheese cloth. Tissue is retained on the cheese cloth while the fluid and sperm pass through. Simple centrifugation of the filtrate yields luminal fluid as the supernatant and sperm as a pellet. Thus, sperm from 12 segments of goat epididymis were obtained.

Since the main interest was in studying changes on the sperm surface it was essential to obtain pure sperm plasma membranes. This was done by sonication and centrifugation at high speeds in sucrose gradients. The identity of the membrane fraction obtained was checked by electron microscopic studies employing phosphotungstic acid as a negative stain. Adenylate cyclase served as an enzyme marker for final confirmation.

Since lipids, carbohydrates and proteins form important constituents of membranes, changes in these constituents were monitored in membrane preparations of sperm collected from twelve different sites of the epididymis. The results indicate that sperm membranes tend to loose substantial amounts of phospholipids, cholesterol, neutral sugars, amino sugars, sialic acid and proteins during epididymal transit. The ratio of cholesterol to phospholipid, however, rose from 0.41 to 0.51. The significance of these findings has been analysed in the light of events happening in the epididymis.

Of the six membrane constituents, sperm surface proteins were selected for detailed examination. These biological molecules exhibit the greatest heterogeneity and some on account of being acquired or modified in the epididymis are suspected to perform unique functions. In order to study the protein profiles of sperm surface it is essential to release and isolate these molecules from the membrane preparations. To achieve this, two ionic detergents (sodium dodecyl sulfate and sodium deoxycholate) and two nonionic detergents (Triton X-100 and Nonidet P-40) were used. Optimal solubilization conditions for each of these detergents were determined worked out.

Analysis by polyacrylamide gel electrophoresis revealed that when ionic detergents were used, the staining intensity of low molecular weight proteins was high. On the other hand, the use of non ionic detergents resulted in better staining of proteins having a molecular weight in the intermediate range. On the whole the solubilization of membrane proteins by sodium dodecyl sulfate was found to be superior in terms of the higher quantity and the number of proteins that could be obtained.

When solubilized proteins from membrane preparations of sperm isolated from 12 sites of the goat epididymal duct were analyzed on SDS-PAGE, several differences could be observed. For a more detailed examination there was need to isolate and purify specific proteins of interest. Commercially available equipment are able to elute proteins from polyacrylamide gels but these methods are not useful for separating a mixture of proteins from these gels.

Therefore an effort was made to design and fabricate an apparatus that could elute proteins during an electrophoretic separation. The assembly made resembled a disc electrophoretic apparatus except that a single large sized gel tube is used with provision of elution towards the bottom. Both low and high molecular weight proteins could be resolved and eluted by altering the percentage of gel. In addition, the performance of the apparatus was governed by the strength of electric field and flow rate of buffer used for elution.

Sperm surface proteins that were solubilized by sodium dodecyl sulfate were initially electrophoresed on 10% slabs of SDS polyacrylamide. When electrophoresis was complete, the slab was cut horizontally such that the top half containing the high molecular weight proteins could be separated from the lower half containing low molecular weight proteins. Each half of the gel was minced separately in appropriate buffer and used as sample to be loaded on the special apparatus designed and tested for elution of proteins from polyacrylamide gel.

From the elution profiles significant observations were made. A 124kD protein was detected from the surface of the caput sperm which was not present among the proteins solubilized from cauda sperm membranes. Instead, an additional protein of 31kD was found in cauda sperm membrane. Polyclonal antibody to the 31kD protein specifically interacted with the 124kD protein, but not any other high molecular weight proteins solubilized from caput sperm. Further, limited proteolysis of both the 124kD and 31kD protein by

cyanogen bromide and chymotrypsin treatment followed subsequently by analysis on polyacrylamide gel showed that certain fragments were common to both these proteins.

These results suggest that the proteolytic cleavage of the 124kD protein results in the formation of the 31kD protein. This transformation is seen as one of the mechanisms involved in the post translational modification of sperm membrane protein. Numerous instances have been reported in the literature where proteins become biologically activated when cleaved. It is proposed from the present study that the 31kD protein might be an activated form that participates in sperm maturation and storage.
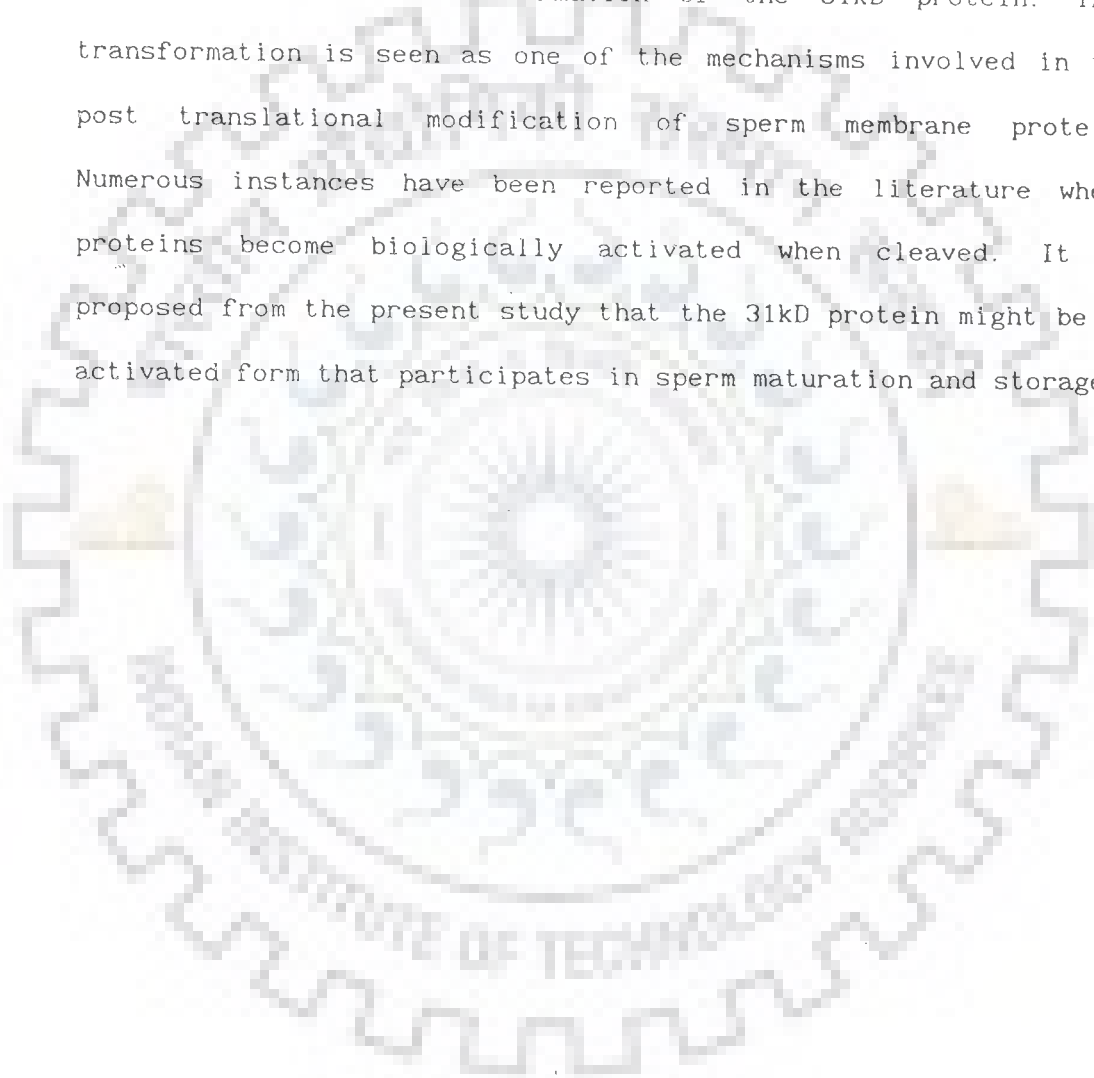
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(NEETI SRIVASTAVA)

INTRODUCTION

Sperm are highly specialized cells destined to accomplish the task of transferring genetic material to the ovum for fertilization. The spermatozoa produced in the mammalian testis are incapable of forward progressive movement and lack the eapacity to fertilize the egg.not inherent qualities of the spermatozoa produced in the mammalian testis. Spermatozoa undergo a gamut of changes during their passage through the epididymis before they become viable, a process referred to as sperm maturation. The importance of these changes could be judged from the efforts made to achieve post-testicular contraception by interfering with maturational changes on sperm. To achieve this it is imperative to have a detailed knowledge about events that occur in the epididymis and exactly how they influence sperm.

It is now well known that the epididymis provides a unique environment for transformation of sperm from the immature to the mature form. The period spent by sperm in the epididymal lumen provides ample time and opportunity for these changes to be effected. The secretory nature of the epididymal epithelium provides the right ingredients at the appropriate time for the maturing sperm. Besides, the epididymis is also able to rapidly dispose several components not required by the sperm. Thus, the sperm is housed in a suitable, protected environment until required for fertilization.

In recent times much effort has been directed towards understanding the mystery behind the phenomena of sperm maturation. Despite the quantum of effort directed towards this

area, certain aspects of this process still remain unravelled. Therefore, an attempt has been made here to study some of the biochemical aspects of the process of sperm maturation.

Using goat as the animal model, sperm membrane changes have been investigated during epididymal transit. From these findings an effort has been made to decipher the mechanisms by which these changes are brought about.

The approach used and the objectives are defined as follows.

- Isolation of sperm from several sites along the epididymis.
- Preparation of sperm membranes and studying the changes in lipid, carbohydrate and protein composition.
- Extraction and solubilization of membrane proteins using detergents.
- Development of a suitable apparatus for purifying and isolating specific membrane proteins of interest.
- Analysis of sperm membrane proteins from sperm samples collected at various sites along the epididymal duct.
- Propose a suitable mechanism for explaining the nature of changes in membrane associated proteins.

LITERATURE REVIEW

2.1 INTRODUCTION

The spermatozoon is a highly specialized cell produced by the seminiferous tubules of the testis (Steinberger and Steinberger, 1975 and Bellve, 1979). In mammals, the spermatozoa share some common characteristics and at least two distinct regions, the head and the tail or flagellum can be recognised (Baccetti and Afzelius, 1976). The head consists of the acrosome, the nucleus and small amounts of cytoskeletal structures and cytoplasmic contents. The acrosome is a large secretory granule that closely surrounds and overlies the anterior end of the nucleus. The sperm nucleus is haploid containing only one member of each chromosome pair and the chromatin becomes highly condensed during late spermatogenesis. The tail contains a centrally placed axoneme which is a highly ordered complex of microtubules surrounded by dense fibres extending from the head to near the posterior end of the axoneme. In addition, the anterior part of the flagellum contains mitochondria wrapped in a tight helix around the dense fibres, and at the posterior end of the tail the fibrous sheath surrounds the dense fibres. The dense fibres and fibrous sheath form the cytoskeleton of the flagellum. These cytoskeletal features appear to have evolved with the development of internal fertilization. The tail, like the head, is closely wrapped by the plasma membrane and contains little cytoplasm. Although in most mammals the structure of spermatozoa conforms to this description, certain species specific variations can also be seen with regard to the shape and size of these parts (Baccetti and Afzelius, 1976 and Roosen - Runge, 1977).

The spermatozoan is basically a carrier for the delivery of genetic material contained in its nucleus to the egg. To accomplish this function effectively, it undergoes a vast array of changes after being produced in the seminiferous tubules of the testis. These changes are morphological, biochemical and physiological in nature and result from a highly complicated process (Mann and Lutwack - Mann, 1981).

2.2 SPERM - ARCHITECTURALLY A UNIQUE TYPE OF CELL

Typically, sperm are stripped down cells, highly compact, streamlined and equipped with a strong flagellum to propel them through an aqueous medium. They lack several cytoplasmic organelles such as ribosomes, endoplasmic reticulum or golgi apparatus, which are unnecessary in the task of delivering the DNA to the egg (Eddy, 1988). On the other hand, numerous mitochondria are strategically placed where they can most efficiently power the flagellum (Mann and Lutwack-Mann, 1981; Eddy, 1988 and Lindemann and Kanous, 1989). Sperm usually consists of two morphologically and functionally distinct regions: the head, which contains an unusually condensed haploid nucleus, and the tail, which propels the sperm to the egg and helps it burrow through the egg coat. Both these regions are enclosed by a single plasma membrane. The DNA in the nucleus is inactive and extremely tightly packed, so that its volume is minimized for transport (Eddy, 1988). At times the chromosomes of many sperm have dispensed with the histones of somatic cells and are packed instead with positively charged proteins (Grimes, 1986; Redi et al., 1982 and Bellve and O'Brien, 1983).

In the sperm head, closely apposed to the anterior end of the nuclear envelope is a specialized secretory vesicle called the acrosomal vesicle. This vesicle contains hydrolytic enzymes that help the sperm penetrate the egg's outer coat (Allison and Hartree, 1970). When a sperm contacts an egg, the contents of the vesicle are released by exocytosis, a process known as the acrosome reaction (Hartree and Srivastava, 1965 and Allison and Hartree, 1970). In many invertebrate sperm this reaction also releases specific proteins for example Bindin that bind the sperm tightly to the egg coat (Vacquier and Moy, 1977).

The sperm tail is a long flagellum whose central axoneme emanates from a basal body situated just posterior to the nucleus. The axoneme consists of two central single microtubules surrounded by nine evenly spaced microtubule doublets (Eddy, 1988). The flagellum of some sperm (including those of mammals) differs from other flagella in that the usual 9+2 pattern of the axoneme is further surrounded by nine outer dense fibres of unknown composition (Eddy, 1988). The dense fibres are stiff and noncontractile, and it is not known what part they play in the active bending of the flagellum which is caused by the sliding of the adjacent microtubule doublets past one another. Flagellar movement is powered by the hydrolysis of ATP, generated by highly specialized mitochondria in the anterior part of the sperm tail called the mid piece (Phillips, 1970).

2.3 SPERM MEMBRANES

It is known that one plasma membrane covers the entire sperm surface, however, five functionally, structurally and biochemically distinct domains have been recognized (Friend, 1984; Holt, 1984; Wolf and Voglmayr, 1984: Villarroya and Scholler, 1986; Wolf et al., 1986 and Bearer and Friend, 1990). The function of each of these domains is specific and ultimately directed to the fusion of the sperm with egg. For this reason the integrity of the domains is generally maintained.

The anterior head or what is referred to as the **acrosomal segment** is involved in the binding of sperm to the zona pellucida. This is associated with the exocytosis of the acrosomal contents, furthering the presentation of the inner portion of the granule membrane as the new surface. This segment overlies the acrosome granule and bears a hexagonal pattern which is shaped during late spermatogenesis (Elias et al., 1979 and Friend, 1984).

The **equitorial segment** is the site on sperm plasma membrane where the external fusion with the egg occurs (Toshimori et al., 1987 and Bearer and Friend, 1982). It also acts as a barrier preventing diffusion between the acrosomal and post acrosomal regions of the plasma membrane lying on either sides of this domain.

The **post acrosomal** segment contains a dense population of intramembranous particles and the basal part of this domain contains prominent cords or bands of particles (Koehler, 1982).

This domain acts as a seal between the head and tail of the sperm thereby maintaining the integrity of the acrosomal domain.

The **midpiece** is characterized by the presence of a pair of centrioles from one of which projects the flagellar microtubules, a neck rich in intermediate filaments and actin, and the circumferentially arranged mitochondria (Friend and Heuser, 1981). It is separated from the head by a striated ring, a membrane specialization of unknown composition. A string of intramembranous particles encircle the midpiece like a necklace.

The **principal piece** has larger intramembranous particles which are basically a staggered double row of still larger intramembranous particles coursing over the ribs of the fibrous sheath, yet another characteristic feature of the principal piece are larger particles arranged in a zipper like fashion. Since this region lies over the tail portion of the spermatozoan, it is believed to be involved in the motility of the sperm and the various types of flagellar movement (Friend and Fawcett, 1974 and Enders et al., 1983).

The five domains of the sperm membrane described above are principally formed in the testis during spermatogenesis and continue to be modified during passage through the epididymis (maturation) and in the female reproductive tract (capacitation).

2.4 THE EPIDIDYMIS

The mammalian epididymis is a highly convoluted duct juxtaposed to the testis. Initially this organ was considered an abandoned child

of the reproductive system (Mann, 1964). But over the past several years the epididymis has slowly come to be recognized as a duct that regulates maturation and storage of spermatozoa (Reid and Cleland, 1957; Cooper, 1986; Amann, 1987 and Robaire and Hermo, 1988). Research activities are now oriented towards studies on the various processes/events occurring within this organ down to the minutest detail.

Despite such efforts, our understanding of the epididymis still remains far from clear. One reason for the lack of proper understanding of the organ's functioning has been the arbitrary nature of segmentation used by researchers investigating events in the epididymis. This has made interpretation of data difficult. Nevertheless, in most cases the epididymis has generally been divided into the caput (head), corpus (body) and cauda (tail). Again, even this system of classification has not been universally adopted since the shape and size of the organ and regional specialization of the epididymis on account of the differential distribution of cell types are not identical among various species of mammals. Thus, for each species of mammal a unique type of segmental nomenclature is currently in use (Amann et al., 1982; Besancon et al., 1985; Marengo and Amann, 1990 and Vreeburg et al., 1990).

In large mammals, like the ram, the epididymis has been conveniently divided into 12 segments (Besancon et al., 1985). Studies of the fluid collected from these sites have provided evidence for the active involvement of the epididymis in processes

like absorption, secretion, metabolism and spermiophagy (Mann and Lutwack-Mann, 1981 and Robaire and Hermo, 1988).

Rete testis fluid (RTF) proteins conjugated to colloidal gold when injected intraluminally at specific sites in the excurrent ducts were found by electron microscopy, to be endocytosed into the ductuli efferentes (Veeramachaneni et al., 1990). This gives an indication that the epididymis is a major extra-testicular site of endocytosis of bulk proteins presents in the RTF. The survival of the androgen binding protein through the efferential duct systems is also indicative that the endocytosis is specific and not indiscriminate in nature (Danzo and Black, 1990a).

Comparison of the protein profiles in luminal fluid collected from various sites of the epididymal duct have shown that the epididymis is capable of secreting a variety of proteins (Koskimies and Kormano, 1975 and Turner, 1979). Besides proteins, the epididymis has been reported to provide several other biological molecules that are essential for sperm maturation (Orgebin-Crist et al., 1975).

In this context, it would not be out of place to mention that a variety of enzymes known to be associated with several metabolic pathways are active in the epididymis. Poulos et al. (1973a,b) and Evans and Setchell (1979) have even demonstrated the presence of a number of intermediates of lipid metabolism in the epididymis.

These and other reports collectively suggest that synthesis and breakdown of biological molecules are routine events that take place in the epididymis. What is interesting are the efforts made to relate these metabolic events with the attainment of functional maturity of sperm (Brooks, 1981a).

There is also fragmentary evidence to suggest that the epididymis could be a site for the disposal of sperm within the male reproductive tract. The quantitative difference in daily testicular production of sperm and daily output of spermatozoa in ejaculated semen indicates that a part of the testicular spermatozoa is eliminated within the male reproductive tract. Selective destruction of dead/abnormal/aging spermatozoa and absorption of degraded products by epithelial cells lining the epididymis or wandering macrophages may be the possible routes for clearing the sperm debris (Amann and Almquist, 1962; Cooper and Hamilton, 1977 and Holstein, 1978). The exact mechanism and extent of elimination is, however, not known.

The variety of animal models used has only complicated our efforts in understanding the physiology of the epididymis. This has primarily been due to two variables: (i) the time spent by sperm in the epididymis and (ii) the composition of luminal fluid along the epididymal duct. But the fact remains that the fluid is a mixture of epididymal secretions: ions (Salisbury and Cragle, 1956); organic acids (Chinoy et al., 1983), sugars (Arora et al., 1975); Glycerylphosphorylcholine (Hinton and Setchell, 1980a), carnitine (Hinton and Setchell, 1980b) and a variety of other

biochemical components. Physiologically the low oxygen tension, low pH, high CO_2 tension, high potassium ion concentration, hypertonicity, the dehydration of sperm during transit and finally presence of metabolic regulators are the factors responsible for the transformation of sperm.

Thus, testicular spermatozoa when exposed to the epididymal microenvironment undergo dramatic changes that lead to their acquiring both motility and fertilizing ability.

The morphological changes that occur in sperm include the shifting of the cytoplasmic droplet from the neck to the distal end of the mid piece (Mann and Lutwack-Mann, 1981 and Kaplan et al., 1984) and change in size of the acrosome (Eddy, 1988). The ability to move forward (Acott and Hoskins, 1978), capacity for head to head agglutination (Dacheux et al., 1983) and increasing net negative charge (Bedford, 1963) are some of the other special traits that are attained.

2.5 SPERM PLASMA MEMBRANE -- BIOCHEMICAL CONSTITUENTS

As mentioned before sperm possess a complex plasma membrane which becomes progressively modified during their journey towards the egg. This is evident from the microscopic and indirect measures used to monitor the chemical and physical changes occurring on sperm membranes. Changes in functional abilities like sensitivity to cold induced damage (Hammerstedt et al., 1979) strongly suggest that the level of organization with respect to membrane lipids in testicular and ejaculated sperm are not the same.

2.5.1 Lipids

Sperm membranes just like membranes from any other type of cell are predominently composed of lipids. But unlike the membranes of most cells, the lipid component of sperm membrane is continuously reorganized starting from the time sperm are produced until they finally meet the egg.

There are several reasons why one can expect dynamic changes in sperm membrane lipids during their sojourn in the epididymis (Grogan et al., 1966). First, testicular sperm acquire both motility and fertilizing ability during passage through this duct. Secondly, the activity of sperm is dramatically reduced to prevent premature acrosomal reaction, and the viability is tremendously enhanced to facilitate storage as they are transported from the caput to the cauda epididymis. Finally, the sperm is prepared for later events such as capacitation (Davis, 1981) and fertilization (Hammerstedt et al., 1979). All these mean that the sperm membranes need to be reorganized from time to time for simultaneously maintaining a high level of stability.

In ram spermatozoa, changes in the cholesterol and phospholipid contents have been reported in the acrosomal portion of the membrane. Data suggests that the molar ratio of cholesterol: phospholipid increases in plasma membrane during maturation (Demel and Dekruff, 1976 and Chapman, 1982). Investigations have also revealed that the head groups and unsaturated fatty acid residues are replaced but without change in the charge of the membrane phospholipid (Schelegel et al., 1986). Further, the bulk of

phospholipid bound fatty acid was primarily palmitoyl acyl groups in caput sperm and docasohexanoyl acyl groups in cauda sperm (Poulos et al., 1973a,b and Evans and Setchell, 1978). Ethanolamine and choline phosphoglycerides represent 70-80% of the membrane phospholipids. During epididymal maturation of sperm the ethanolamine phosphoglycerides decrease relative to choline phosphoglycerides (Parks and Hammerstedt, 1985).

2.5.2 Carbohydrates

It has been known for a long time that spermatozoa from the seminiferous tubules and proximal regions of the epididymis are incapable of binding to the zona pellucida and fertilizing the egg. The mammalian epididymis is thought to provide the specific intraluminal environment for the morphological and biochemical modification necessary to produce functionally mature spermatozoa. The sperm plasma membrane is a vital component during early events in fertilization and undergoes extensive biochemical changes as spermatozoa move from the caput to the cauda region of the epididymis. Carbohydrate moieties are a major component of sperm surface that are modified and processed for interaction with egg. The abundance of glycohydrolases and glycosyltransferases in the epididymal lumen makes this possible (Conchie et al., 1959; Jones and Glover, 1973 and Chapman and Killian, 1984). Changes in carbohydrate structures have been monitored by surface labeling studies employing lectin binding (Magargee et al., 1988), borotritide reduction of oxidized galactose or sialic acids in glycoprotein oligosaccharides (Voglmayr et al., 1983), lactopero-

xidase-mediated iodination of tyrosine residues in surface molecules (Ji et al., 1981), colloidal iron hydroxide (Holt, 1980) and cationized ferritin (Nicolson et al., 1977).

In ram sperm, surface labeling studies for carbohydrates showsthat in sperm from caput, corpus and cauda epididymis, Concanvalin A (mannose positive) bound weakly over the entire surface with little change associated with maturation in the epididymis. Glycine Max Lectin (N-acetyl galactosamine positive) binding to sperm surface was relatively stronger to the caput sperm, being concentrated apical ridge portion of the cell. Extensive reduction of the labeling intensity has been observed during epididymal transit. Ricinus communis lectin, Arachis hypogea lectin and Erythrina cristagalli lectin (galactose positive) generally gave similar results, where initially strong binding to the entire sperm surface was observed which decreased (over all parts of the surface except anterior head) during epididymal transit. Triticum vulgaris lectin (Sialic acid positive) binding initially was weak but increased after sperm passed through the epididymis (Magargee et al., 1988).

The net negative charge on spermatozoa increases during epididymal transit which has been attributed to sialic acids (Yanagimachi et al., 1972 and Moore, 1979). Quantitative analysis of sperm surface sialic acid residues indicates that in most species there is an overall decrease in the content of sialic acid during maturation of sperm in the epididymis (Gupta et al., 1974 and Arora et al., 1975). Holt (1980) suggests that the acrosomal

sialic acids contribute a major portion of total sialic acids found on sperm (Hartree and Srivastava, 1965). It has been suggested that because of a reduction in the size of acrosome during epididymal transit the sialic acid is perhaps lost with other acrosomal components (Bedford, 1965 and Jones 1971). In certain other mammals, however, the sialic acid content during epididymal transit has been found to increase (Rajalakshmi and Prasad, 1968). This has been attributed to an increased sialylation of new surface components in the form of sialoglycoproteins (Tulsiani et al., 1993).

Hall and Killian (1987) report a drastic reduction in the amount of total non-amino carbohydrate content of rat spermatozoa during epididymal transit. Coincident with this is an increase in the level of glycohydrolases in the epididymis. This change in carbohydrate content can largely be attributed to post translational modification of sperm glycoproteins by deglycosylation. Tulsiani et al., (1993) have reported high levels (~80%) of glycosyltransferases in the epididymal fluid with some of them exhibiting maturation related changes in the epididymis.

Glycoproteins are a major component of the sperm surface and extensive remodelling in the oligosaccharide component has been reported during epididymal sperm maturation. Mouse spermatozoa specifically glycosylate high molecular weight components, while low molecular weight glycopeptides serve as substrates for specific soluble epididymal fluid galactosyl transferases (Shur

and Hall, 1982). Glycoprotein: galactosyl transferases transfer galactose from UDP-Gal to terminal N-acetyl glucosamine residues on to the free sugar moieties to produce N-acetyl lactosaminyl linkages (Schachter and Roseman, 1980).

Changes in sperm surface glycoproteins may also be brought about by a degradation process in which terminal galactosyl and galactosaminyl residues are cleaved from the oligosaccharide (Jones et al., 1981 and Voglmayr et al., 1983). The activity of two enzymes, α-mannosidase and β-N acetylglucosaminidase is high in epididymal fluid and spermatozoa. It has been noted that both mannose and N-acetyl glucosamine are major components of the acrosomal polysaccharide complex of spermatozoa (Hartree and Srivastava, 1965).

Alterations in the terminal carbohydrate components of the glycoproteins by deglycosylation results in alteration in protein size and/or structure. This may lead to changes in tertiary conformation viscosity and charge, and trigger membrane related events leading to the acquisition of motility and fertilization capacity.

The modification of surface carbohydrates may be important in capacitation, acrosome reaction and sperm - egg interaction. Specific sugar configurations on androgen binding proteins are responsible for their presence in the epididymal fluid as they are not absorbed in the rete testis, where most testicular fluid proteins are absorbed (Danzo and Black, 1990a). Presence of

adequate amounts of sialic acid on sperm surface is essential for their survival in the female genital tract (Czuppon, 1984). The human and rabbit testosterone binding proteins and the rat androgen binding protein have been found to be identical and discrepancies in the molecular weight and subunit ratios have been attributed to their differential glycosylation, which may be responsible for their secretion and/or mechanism of action (Danzo et al., 1989a,b). Certain types of sugar moieties may serve a protective role by masking the sites on sperm that are recognized by macrophages (Czuppon, 1984 and Toshimori et al., 1990a,b).

2.5.3 Proteins

In addition to lipids, proteins are important constituents of sperm membranes, and slight variations in this component result in substantial changes in function of this haploid cell. In fact, small changes lead to the complete transformation of the sperm to the mature form and there is ample opportunity for this to be accomplished in the epididymis.

Dacheux and Voglmayr (1983), based on tracer studies followed by protein analysis, categorized sperm maturation in the ram epididymis into three phases. Rapid sperm surface changes were observed in Phase I which was confined to the caput epididymis. Major components in the 78 to 115 kD protein zone of the testicular sperm surface were found to disappear. Simultaneously, several low molecular weight components (from 17 to 65 kD) made their appearance. Other investigations revealed that the

endocytosis of the majority of proteins of testicular origin taked place in the epididymis.

Phase II was recognised as a relatively quiescent phase restricted to the corpus. Interestingly Phase III, which occurs in the cauda, is characterized by the predominant appearance of several new components, a 24kD protein and increased labeling intensity of several preexisting proteins (97, 65 and 41 kD). In addition, the protein profiles in the epididymal luminal fluid of the caput, corpus, and cauda also suggest that the epididymis actively participates in bringing about the changes in sperm protein in three ways:

i)   Modification or realignment of existing components

ii)   Addition of new components

iii) Loss of certain components.

Several mechanisms have been proposed to explain each category of sperm surface changes, some of which are highlighted. The epididymis is well equipped with an elaborate enzymatic machinery to effect these changes.

2.6 MODELS FOR SPERM MATURATION

2.6.1 Protein Carboxymethylation/Demethylation

The discovery of two enzymes, protein carboxymethylase and protein methylesterase, in the epididymis forms the basis of suggesting that carboxymethylation and demethylation could be the possible mechanisms that contribute to sperm surface changes (Bouchard et al., 1980).

The enzyme protein carboxymethylase modifies free carboxyl groups of glutamate and aspartate residues leading to altered charge and function of proteins (Paik and Kim, 1980). The enzyme protein methylesterase reverses this reaction (Gagnon and Heisler, 1979; Paik and Kim, 1980 and Diliberto, 1982). The activity of protein carboxymethylase has been found to increase 4 to 5 fold during epididymal maturation. The corresponding ratio of methyl receptor proteins: protein carboxymethylase activity was 10 times higher in sperm collected from cauda epididymis than in testicular sperm. A slightly different picture has been reported with respect to protein methyl esterase. The activity of this enzyme in testicular sperm is low and a rapid (~4 fold) increase has been reported in the caput sperm. This activity, however, gradually decreases once again to very low levels in the caudal spermatozoa. The protein carboxymethylase activity has been primarily localized to the sperm tail and is suspected to play a role in regulating sperm motility (Bouchard et al., 1980). Little follow up work has been done in this area and none of the sperm surface proteins have been reported to undergo carboxymethylation or demethylation so far (Gagnon et al., 1984).

Proteins when methylated are known to become less soluble in aqueous media and therefore tend to adhere to membranes spontaneously. This mechanism may be of significance in the transfer of epididymal proteins to the sperm surface (Jones, 1989b).

2.6.2 Phosphorylation/Dephosphorylation

Lindemann (1978) showed that addition of c-AMP to inactivated bovine sperm produced an increase in beat frequency and proportion of motile cells. A similar activation of sperm was observed when they were exposed to certain compounds that inhibited c-AMP phosphodiesterase.

Since c-AMP phosphodiesterase is responsible for the conversion of c-AMP to 5'-AMP, the twitching observed in immotile caput sperm was attributed to an increased level of c-AMP (Hoskins et al., 1975 and Lindemann and Kanous, 1989).

In most eukaryotic cells, c-AMP is known to facilitate protein phosphorylation through activation of c-AMP dependent protein kinases. The occurence of c-AMP dependent protein kinase has been shown in sperm of several species, and in fact represents a significant portion of protein present in sperm extract (Garbers et al., 1973; Biswas and Majumdar, 1982 and Noland et al., 1986). A number of protein substrates for c-AMP-dependent kinase have now been identified in both sperm and soluble fractions from bovine sperm homogenates (Huacuja et al., 1977; Chulavatnatol et al., 1982 and Noland et al., 1984). Phosphorylation is also known to modify the tertiary conformation of proteins (Tash et al., 1984). This supports the contention that phosphorylation and dephosphorylation are important mechanisms for regulation of sperm motility.

2.6.3 Sulfhydryl Proteins and Sperm Maturation

Investigations have revealed that both testicular and caput epididymal spermatozoa are rich in sulfhydryl (SH) groups. The oxidation of sperm sulfhydryl to disulfides (S-S) occurs as spermatozoa migrate through the epididymis. Thus, when sperm reach the cauda epididymis they become rich in disulfide groups. Two enzymes, glutathione peroxidase and glutathione reductase, are believed to bring about these transformations (Calvin et al., 1973). The glutathione peroxidase (Glutathione H_2O_2 : oxido reductase) catalyses the oxidation of glutathione from the reduced (GSH) to the disulfide (GSSG) form and at the same time reduces H_2O_2 tc H_2O. On the other hand, glutathione reductase NAD(P)H: oxidised glutathione oxidoreductase reconverts GSSG to GSH. Both these enzymes have been widely implicated in the maturational modification of sperm surface sulfydryl proteins (Brown et al., 1977 and Smith et al., 1979). Yet another enzyme involved in sulfhydryl related maturation is sulfhydryl oxidase which is secreted in the male reproductive tract (Chang and Zirkin, 1978). This enzyme catalyses the oxidation of 2-mercaptoethanol and other low molecular weight compounds to the corresponding disulfides at the expense of molecular oxygen accompanied by formation of hydrogen peroxide.

Moreover, it has been noticed that much of the sulfhydryl oxidation occurs in the outer dense fibres of the sperm tail, the connecting piece, the fibrous sheath and the outer mitochondrial membrane. The oxidation of SH groups of sperm proteins to S-S

occurs concurrently with maturational processes such as acquisition of forward progressive motility and fertilizing ability. Thus, the beat pattern of sperm tail changes during epididymal maturation from a flexible to a more rigid type (Bedford et al., 1973; Bedford and Calvin, 1974 and Baccetti et al., 1976). Another interesting finding comes from caput sperm of hamsters in which forward progressive motility is prevented by a $90 - 180^{\circ}$ bend when diamides are added to the incubation mixture suggesting that sulfhydryl proteins are involved (Cornwall et al., 1988 and Cornwall and Chang, 1990). There is evidence to suggest that sulfhydryl protein may aid in the attachment of epididymal luminal fluid proteins to the sperm (Brooks, 1985). Changes in the oxidation state of sulfhydryl groups perhaps regulate the attachment of proteins bearing surface thiol groups (Hoskins et al., 1975; Lea et al., 1978; Faye et al., 1980; Cuasnicu et al., 1984; Klinefelter and Hamilton, 1985 and Topfer-Petersen et al., 1990a).

2.6.4 Glycosylation/Deglycosylation

The role of carbohydrate moieties in cellular function has been increasingly publicised in recent years. Preliminary studies showed that the labeling intensity of certain glycoproteins on sperm is considerably reduced during passage through the epididymis. Three reasons could possibly account for the observations made: (i) complete removal of glycoprotein from sperm surface, (ii) removal of terminal sugars such as galactose or galactosamine, and (iii) masking of labeling sites by addition of

new components. Evidence for all these three events have been presented in literature (Hamilton and Gould, 1982; Brown et al., 1983; Hamilton et al., 1986; Goldin et al., 1989 and Srivastava and Olson, 1991).

The abundance of several enzymes like glycosyltransferes and glycosidases in the epididymis, lend added support to this contention (Skudlarek and Orgebin-Crist, 1986; Hall and Killian, 1987 and Tulsiani et al., 1993). The mechanism for the formation of sperm cell surface glycoproteins may involve galactosyltransferase present in the rete testis fluid and epididymal fluid which may glycosylate epididymal secretory proteins which are later adsorbed by the sperm surface. α-lactalbumin, a component modifying the substrate specificity of galactosyltransferase has been found in high concentrations in the rat epididymis indicating the highly active state of the enzyme in this organ (Hamilton, 1980 and Jones and Brown, 1982). Hall and Killian (1987) have reported the presence of a large number of glycosidases present on sperm surface and a regional difference in their activities. β-glucosidase, β-N-acetylglucosaminidase, β-galactosidase and β-N acetyl galactosaminidase all exhibit high levels of activities in the corpus epididymis. These glycosidases are apparently secreted by the epididymal epithelium into the lumen where they are bound to spermatozoa. The activity of two enzymes, α-mannosidase and N-acetyl glucosaminidase, is high in the epididymal fluid and spermatozoa. It has also been observed that both mannose and N-acetylglucosamine are major components of

the acrosomal polysaccharide complex of spermatozoa. These enzymes are subsequently involved in a variety of membrane mediated events leading to fertilization.

The changes in size and/or structure of sperm glycoproteins brought about in the epididymal duct may have an impact on the physiochemical properties such as tertiary conformation, viscosity and charge. Eventually several membrane related events might even be triggered leading to the acquisition of motility and fertility. There are also instances where differences in the glycosylated state of molecules have been suggested to play a role in the secretion and/or mechanism of action of glycoproteins.

2.6.5 Proteolytic Cleavages

Another interesting set of post testicular maturational changes occurring on sperm surface involves the proteases. Proteases have been detected in the epididymal lumen, the tissue and also on the sperm. The distribution of a wide range of proteases in epididymis may also be indicative of their role in sperm surface protein modification. Certain high molecular weight components present on the caput sperm are notable by their absence from the cauda sperm. Concurrent with this is the appearance of certain low molecular weight proteins in the caudal sperm. This suggests that transformations involve proteases.

Acrosin, an acrosomal protease, has been widely documented in literature (Zaneveld et al., 1975; Brown et al., 1975; Hardy et al., 1987 and Topfer - Petersen et al., 1990a). This enzyme is a

serine protease. It is packaged as a high molecular weight component (proacrosin) on the testicular sperm. It undergoes autoproteolysis in the epididymis to yield the active low molecular weight component acrosin (Hardy et al., 1987 and Topfer - Petersen et al., 1990a). This enzyme plays a crucial role in egg-sperm interaction by dissolving the zona pellucida following its release from the acrosome after the acrosomal reaction (Dunbar et al., 1985 and Topfer-Petersen et al., 1990a,b,d).

A metallo -protease on human sperm has also been reported (Olivares et al., 1993). It is believed that the enzyme has a high specificity for Immunoglobin A, thereby enabling the sperm to evade the local immune response in the female genital tract. But there are other proteases that are produced in the epididymis and act on the sperm within the male reproductive tract.

Rabbit epididymis has a specific steroid receptor active protease. This protease is found in the adult epididymal cytosol and the nucleomyofibrillar fraction. It is highly tissue specific, being localized to the caput epididymis. It acts on the functional estrogen receptors present in the epididymis and removes their nucleotide - binding sites (Danzo, 1986 and Hendry III and Danzo, 1986).

PH-30 a guinea pig sperm protein that has been found to be involved in egg-sperm interaction, consists of two immunologically distinct α-and β-subunits. Proteolytic processing of pre-β occurs during passage through the epididymis and appears to occur in at

least two steps. In the distal corpus, two bands of decreased molecular weight are seen, pre β and a band that comigrates with $\beta_{1,2}$ of mature epididymal sperm. Final processing of pre β which leads to the appearance of β_3 occurs either in/or during transit to the proximal cauda epididymidis. This indicates that proteolysis plays an important role in sperm maturation as PH-30 is involved in explasmic fusion events (Blobel et al., 1990).

Yet another protein is CE9, which is a posterior tail domain specific integral plasma membrane glycoprotein of the rat testicular spermatozoa. During epididymal maturation CE9 undergoes endoproteolytic processing and then redistributes into the anterior tail plasma membrane domain of spermatozoa (Nehme et al., 1993).

A 135 kD protein purified from cauda epididymal fluid gave a monoclonal antibody which reacts with a 27 kD protein on sperm surface, indicating that this sperm surface antigen has been derived from the epididymal protein after proteolytic digestion (Okamura et al., 1992).

Another important aspect of the action of proteases on sperm surface proteins is their regionalization to specific sperm surface domains. The regionalization of sperm surface proteins could occur by two mechanisms. In the first mechanism, the proteolysis of specific sperm surface components is responsible for their localization in specific domains. In the guinea pig testicular sperm, PH-20 is found on the whole sperm cell while

PH-30 and AH-50 are found on whole head surface. In the fully differentiated cauda sperm, PH-20 and PH-30 are restricted to the posterior head domain, while AH-50 is confined to the anterior head domain. The second mechanism for regionalization is based on events being triggered by the proteolytic cleavage of sperm surface proteins, in which the cleavage acts as an extracellular signal which could be transduced across the plasma membrane and initiate cytoplasmic events, resulting in localization (Cowan et al., 1986, 1987; Primakoff et al., 1988, Blobel et al., 1990 and Phelps et al., 1990).

Thus, proteases can be envisaged to play a critical role in sperm maturation both directly by events occurring on sperm surface and indirectly by the changes brought about by them in the epididymal luminal proteins, for example the epididymal estrogen receptor.

2.7 PROTEINS WITH SPECIFIC FUNCTION

By now, it is clear that proteins are one category of molecules present on the sperm surface that change during maturation. Several attempts have been made to identify these specific proteins and decipher their functions. A number of such proteins have been described in the literature, the most important of which are reviewed.

2.7.1 Acrosin

Acrosin (EC 3.4.21.10) is a serine proteinase of trypsin like specificity. It has carbohydrate binding sites and by specific and limited proteolysis of the glycoprotein matrix aids in

penetration through the zona pellucida (Parrish and Polakoski, 1979; Kennedy and Polakoski, 1981; Dunbar et al., 1985; Urch et al., 1985; Hardy et al., 1987 and Topfer - Petersen et al., 1990a,d).

In boar, acrosin is synthesized in early round spermatids as a single chain polypeptide form proacrosin, and stored in the acrosomal vesicle overlying the anterior part of the head. Proacrosin is converted to its active form by limited self proteolysis at the time of the acrosome reaction, and is discharged from the sperm in the proximity of the egg. The mature enzyme consists of a 23 amino acid residue light chain covalently linked by two disulfide bridges to the heavy chain (Mw 37,000) containing the active centre. It has ~55% homology in sequence to serine proteinases, including the location of the catalytic triad necessary for proteolytic activity. Its unique feature is the 125 residue C-terminal extension containing a stretch of 23 consecutive proline residues. The purified boar sperm acrosin migrates as a 55/53kD doublet when subjected to SDS-PAGE (Topfer Petersen et al., 1990a). The predominant proacrosin in rabbit testis migrates as a 68,000Mw band on SDS-PAGE and on gel filtration it has a Mw of 73,000 (Mukerji and Meizel, 1979). The guinea pig testicular proacrosin migrates as a 62kD protein on SDS-PAGE. Human sperm procrosins have been found to have molecular weights of 75 and 42kD (Tobias and Schumacher, 1977). Proacrosin from different sources, exhibiting variations in molecular weights, fall under

three major categories: a) 55kD doublet, b) larger variants with Mw > 60kD and c) a smaller 43kD variant.

2.7.2 Acrosome Stabilizing Factor (ASF)

This protein, purified from rabbit sperm, is capable of reversible decapacitation in vivo and blocks the acrosome reaction in vitro (Eng and Oliphant, 1978). It is a 260kD glycoprotein composed of two subunits, each of 92 and 38kD. The larger and smaller subunits exhibit very high content of a complex, high mannose, N-linked oligosaccharides. It has multiple isoelectric points indicating charge heterogeneity (Thomas et al., 1984). The acrosome stabilizing factor (AFS) is synthesized and secreted by the principal cells in the corpus epididymidis. The caput has almost undetectable levels of ASF while the levels in the corpus, cauda and vas deferens were 880, 3363 and 3236 μg/ml respectively. The ASF has been found to be highly species specific (Reynolds et al., 1989).

2.7.3 Androgen Binding Protein (ABP)

This is a protein localized to the adluminal region of seminiferous tubules of rat testis and the secretory granules of cultured sertoli cells (Attramadal et al., 1981 and Feldman et al., 1981). Androgen binding proteins have been demonstrated in the epididymis and/or testis of human (Hsu and Troen 1978; Danzo et al., 1989a and Danzo and Black 1990b); rabbit (Danzo et al., 1974, Danzo, 1986; Danzo et al., 1989b and Danzo and Black 1990b); rat (Schmidt et al., 1981; Danzo and Eller, 1985; Danzo and Black, 1990a and Turner and Roddy 1990); guinea pig (Danzo et al., 1982);

hamster (Holland et al., 1987) and sheep (Carreau et al., 1979; 1984a,b). This protein migrates as a single band on PAGE with a sedimentation equilibrium based molecular weight of 100,000. On SDS-PAGE, ABP moves as a doublet of 41,000 and 47,000, the quantitative ratio of the two being 1:3. ABP is a leucine - rich protein. The non polar amino acids account for 51%. The carbohydrate content is 25%. An interesting feature of ABP is that it is not retained in the rete testis despite the fact that a major bulk of the testicular fluid is absorbed there. The oligosaccharides on rat ABP may be important in targeting it for secretion, and/or they may be involved in regulating its absorption in the excurrent duct system (Danzo and Black, 1990a,b). The oligosaccharides may also prevent the degradation and/or uptake of ABP prior to its reaching the epididymis.

The role of androgen binding proteins is not well established. It has been implicated in the maintenance of spermiogenesis by testosterone transfer. Alternatively, it may be important in controlling steroid concentrations in the epididymis. With speculations of binding of non steroidal compounds to ABP, it is possible that it may reduce progressive motility and sperm-egg binding ability of caudal sperm (Hardy et al., 1987).

2.7.4 Immobilin

This is a highly viscoelastic mucus like glycoprotein isolated from cauda epididymal fluid and believed to be synthesized in the caput epididymis (Usselman and Cone, 1983 and Usselman et al., 1985). It has a Mw > 10^6 Daltons and contains 56% carbohydrates,

with low or undetectable levels of mannose, xylose and uronic acid. The major carbohydrate components are galactose, N-acetyl glucosamine and N-acetyl galactosamine arranged in short oligosaccharide chain (about 4 to 20 monosaccharide per chain) linked by O-glycosidic linkage. A notable feature is the absence of sialic acids. The core protein molecule is pronase resistant. The synthesis of immobilin is restricted to the principal cells of the caput epididymis and is secreted by them into the lumen of the tubules (Ruiz-Bravo, 1988).

The function of immobilin is based on its high viscoelasticity, which keeps the sperm immobile in cauda epididymis. It may also prevent the shearing of sperm during ejaculation (Cardullo and Cone, 1986).

2.7.5 Binding Proteins

Mammalian sperm possess specific components in the plasma membrane that are involved in the binding of these cells to the zona pellucida. Proteins exhibiting such binding activites have been identified in mouse (Shur and Hall, 1982 and Jones, 1989a), guinea pig (Cowan et al., 1986) and rabbit (O'Rand et al., 1985).

Jonakova et al., (1991) report a 15kD protein purified from ejaculated boar spermatozoa with zona pellucida binding and haemagglutinating activities. The egg-sperm interraction brought about by the 15kD protein is inhibited by fucoidan, and the N-terminal sequencing indicates sequence homology to the sea urchin sperm protein bindin (Vacquier and Moy, 1977). The 15kD

protein has unusually high levels of polar residues, predominantly serine and glycine. There is a total lack of methionine residues and it has two disulfide bridges in its tertiary structure. This glycoprotein interestingly contains 4 mol glucosamine per mol glycoprotein, but does not contain any galactosamine, indicating the presence of N-glycosidically linked sugar chains.

Boar sperm plasma membranes contain an integral 55kD protein called Adhesion protein (AP_z) involved in the adhesion of sperm to the zona pellucida. This interaction is, however, not mediated through either proteolytic or galactosyl transferase activity (Peterson and Hunt, 1989). It is first expressed in corpus epididymis and attains complete expression in the cauda epididymis with a significant portion of the protein being integral to the cauda epididymal sperm membrane.

2.7.6 Antagglutinin

This is a factor purified from the cauda epididymal fluid of boar and ram that prevents dilution induced agglutination of immature sperm cells (Dacheux et al., 1983). It has been demonstrated that antagglutinins exhibit a remarkable lack of species specificity. It has been observed that the diluted sperm suspensions from corpus epididymis showed head to head agglutination. This agglutination was inhibited by the addition of cauda epididymal plasma.

Dacheux and Dacheux (1989) report that antagglutinin is an androgen regulated protein synthesized by the principal cells of

the caput and corpus. A report by the same authors suggests that the antiagglutinating factor concentration either increases in the cauda epididymal fluid or else this factor is sequestered on the sperm surface itself (Dacheux and Dacheux, 1988). The exact function of antagglutinins is undefined.

2.7.7 Bindin

This 30.5kD protein was detected in sea urchin sperm and found to be responsible for the binding of sperm to the vitelline layer of egg (Vacquier and Moy, 1977). Experimental results suggest that bindin may act by binding to carbohydrate receptors of the vitelline layer glycoproteins (Miller and Ax, 1990). Bindin, purified from different sea urchin species, exhibits a lack of cross reactivity, indicating a high degree of species specificity.

2.7.8 Forward Motility Protein

This is a protein purified from bovine seminal plasma which initiates forward motility in caput spermatozoa in the presence of a c-AMP dependent phosphodiesterase inhibitor (Acott and Hoskins, 1978 and Acott et al., 1979). It exhibits multimeric forms when fractionated on the basis of charge and molecular weight sieving under denaturing conditions. This protein on molecular sieving has a molecular weight of 37.5kD.

The enzymatic studies of this protein indicate a glycoproteinaceous nature. Forward motility protein has been conclusively shown to be an extra accessory gland protein. It also exhibits a

lack of species specificity as seen in studies conducted on monkey, man, dog, rabbit, pig and elephant (Acott et al., 1979). The mode of action of the forward motility protein is said to be based on its ability to induce accumulation of cyclic-AMP intracellularly in the sperm (Brandt and Hoskins, 1980).

2.7.9 Calcium-Binding Proteins

Calcium plays a critical role in regulating sperm motility and acrosome reaction. So far, calmodulin has been identified as the only major Ca^{2+} binding protein in sea urchin and mammalian sperm. Recent reports, based on studies on boar sperm plasma membrane, identify six major proteins with molecular weights 30, 35, 38, 42, 52 and 66kD exhibiting strong binding to calcium (Rufo et al., 1984; Lewis et al., 1985 and Peterson et al., 1989).

2.7.10 Rat Decapacitation Factor

A mouse spermatozoal factor present on its surface is found to inhibit fertilizing ability in a reversible manner (Fraser, 1984 and Fraser et al., 1990). This factor can be removed from the sperm surface by high speed centrifugation, which gives highly fertile gametes (Bedford and Chang, 1962). The addition of this factor results in gametes with poor fertility. This factor is an anionic polypeptide with a Mw of ~40,000 as determined by gel filtration. It is stable to heating for 15 minutes at $100^{\circ}C$, and resistant to proteases at pH 8.0. But at pH 5.0, it looses its activity. Experimental results also indicate the absence of high levels of mannose and N-acetylglucosamine.

2.7.11 Prealbumin Epididymal Specific Protein (PES)

This is a 64kD protein which is androgen dependent. It has been found to have interspecific epitopes which are expressed both in the epididymal epithelium and preacrosomal area of sperm plasma membrane in ram and rat (Fournier Delpech et al., 1988). The antibody to PES 64kD cross reacts with rat PES 32kD and with proteins D and E (Wong and Tsang, 1982 and Brooks, 1982). The antibody also reacts with two human seminal plasma proteins which migrate in the post albumin area under non denaturing electrophoresis. Amino acid analysis of PES 64kD shows a relative abundance of asparagine and serine/threonine which could support N-glycosylation and O-glycosylation respectively (Fournier Delpech et al., 1988). The interspecific epitopes of ovine PES 64kD protein have been speculated to be glycoconjugates. Another interesting feature is the lack of reactivity of anti PES 64kD with the testicular tissue, indicating that PES 64kD is an epididymal protein.

2.7.12 Sperm Maturation Antigen

This is a sialoglycoprotein with a molecular weight of 54kD, secreted by the clear cells of the mouse epididymal epithelium; the antigenic determinant, recognized by MAb T21, is masked initially by sialic acid residues (Toshimori et al., 1988). The expression of the antigenic determinant on the sperm surface during epididymal maturation apparently involves desialylation. The MAb T21 also recognises two (31kD and 34kD) antigens; however, their relation to the 54kD antigen is not known. The T21 antigen

may serve as an antiagglutinin. It has also been found to be the major sperm plasma membrane glycoprotein. The presence of terminal sialic acids on T21 could be responsible for its antiagglutinating property. The addition of the 54kD sialoglycoprotein to the mouse caput sperm prevents tail-to-tail agglutination (Toshimori and Eddy, 1985 and Vernon et al., 1987).

2.7.13 Sulfated Glycoprotein - 1 (Saposin Precursor)

This is a heavily glycosylated and sulfated protein of 70Mw secreted by sertoli cells (Sylvester et al., 1989). This protein has also been referred to as Band -4 (Sylvester et al., 1984). SGP-1 shares substantial sequence similarity with human prosaposin, the precursor of lysosomal saposins (O' Brien et al., 1988). Saposins are known to enhance the activity of lipid modifying enzymes presumably by solubilizing the lipids (Morimoto et al., 1988). This protein is present in secondary lysosomes of sertoli cells, and also in the luminal fluid of seminiferous tubules and epididymis. Though not much is known of the exact function of this protein, it is supposed to be involved in degradation of lipids in residual bodies and may also assist in modification of membrane lipids during sperm maturation.

From the foregoing account, it is clear that the events taking place in the epididymis have attracted the attention of several investigators interested in male reproductive physiology. There seems no doubt that sperm maturation and storage are highly complicated processes. Several theories have been proposed to explain specific biochemical events that occur within the

epididymis, particularly with reference to proteins of sperm membranes. It is clear that analysis of epididymal and spermatozoal proteins is not a simple affair since there is tremendous diversity in the type of proteins that have been identified.

There is now need to study the protein profiles in a systematic manner. To achieve this, sperm need to be isolated from various epididymal sites, its membranes purified, membrane proteins solubilized, and special techniques for protein isolation and analysis developed. The matter embodied in this thesis is a consolidation of such an effort.

3.1 INTRODUCTION

On a number of previous occasions it has been amply demonstrated that sperm is produced in the testis, matures in the epididymis, and is then stored in a viable form until ejaculation (Orgebin - Crist et al., 1975 and Mann and Lutwack - Mann, 1981). It has also been confirmed that sperm undergo morphological, biochemical and physiological changes as they transit the epididymal duct (Bedford, 1973; Amann, 1987 and Robaire and Hermo, 1988). In fact, in mammals the epididymis has been identified as the major region of the male reproductive tract that provides the ideal milieu and microenvironment for maturation of sperm (Howards et al., 1975). In addition, it seems that the time spent by sperm in this organ, provides adequate opportunities for it to achieve functional maturity in terms of acquiring motility and fertilizing ability. Thus, as an organ that supports sperm maturation, the epididymis has captured the interest of many researchers.

Until now, most studies have dealt with the epididymal tissue, luminal fluid and sperm as one entity. This has made interpretation of data relating to any one compartment rather difficult. Over the years, several efforts have been made to isolate sperm from the epididymis using techniques of micropuncture (Howards et al., 1975) and retrograde flushing (Jones et al., 1981) with varying degrees of success. In micropuncture, the yield is remarkably low, while retrograde flushing can be used to obtain sperm only from the cauda epididymis. Therefore, there is a pressing need to develop

methods suitable for maximal extraction of sperm from any region of the epididymis.

It is logical to expect that acquisition of fertilizing ability could stem from changes effected on the sperm surface. Recognition of ova is a prelude to successful egg-sperm interactions, and depends to a large extent on the type of molecules present on the surface of both the gametes. Studies of the sperm plasma membrane could thus provide an excellent model where membrane changes could be related to this important biological function. It would be ideal, therefore, to look for changes in the composition of sperm collected from various sites along the epididymis.

In order to investigate what happens to the sperm surface, efforts have been directed towards obtaining sperm free of any other contaminating cells from twelve sequential segments of goat epididymis and purifying their plasma membranes. By comparing the changes in the lipid, protein and carbohydrate composition of these sperm membrane preparations, an attempt has been made to identify and understand the possible role of these molecules in epididymal sperm maturation.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of Sperm

Goat epididymis (weighing between 10 to 14g) are collected from the local abattoir and transported to the laboratory in ice within one hour of slaughter. The epididymis is cleared of adhering adipose and connective tissue and rapidly frozen. For the purpose of standardization of procedures used for isolation of sperm, the epididymis is divided into three major segments: the caput (head), the corpus (body), and cauda (tail). Each segment is sliced into 1mm thick sections using a razor blade. The sliced tissue is then transferred to a filtration flask. Sufficient amount of 20mM HEPES buffer, pH 7.0, containing 1mM PMSF is added to submerge the tissue slices (100mg tissue per ml buffer). The flask is corked and the side arm connected to an aspiration assembly with provision for measuring and controlling the extent of vacuum. The tissue slices are exposed to desired vacuum (50, 100, 200, 250 and 300mmHg) for varying periods of time (0.5, 1.0, 1.5, 2.0, 3.0, 5.0 min) with gentle swirling. The suspension is poured through two layers of cheesecloth. The tissue slices retained on the cheesecloth are recovered. The filtrate which is a suspension of sperm, luminal fluid and possibly cellular contaminations is centrifuged at 3000xg for 15 minutes on a table top centrifuge. The supernatant contained the luminal fluid, and the sperm form the pellet. Thus, the procedure is useful for the separation of epididymal tissue, luminal fluid and sperm.

Fig. 3.1   Scheme for Separation of Tissue, Fluid and Sperm from the
            Epididymal Duct.

Epididymal slices 1mm thick

Suspended in 20mM HEPES buffer pH 7.0
containing 1mM PMSF

Transferred to filtration flask and subjected
to vacuum

Filtered through two layers of cheesecloth

Epididymal   Luminal fluid + Sperm
Tissue   (Filtrate)
(Residue)   Centrifuged at 3000xg
                        for 15 min.

            Luminal fluid   Sperm
            (Supernatant)   (Pellet)
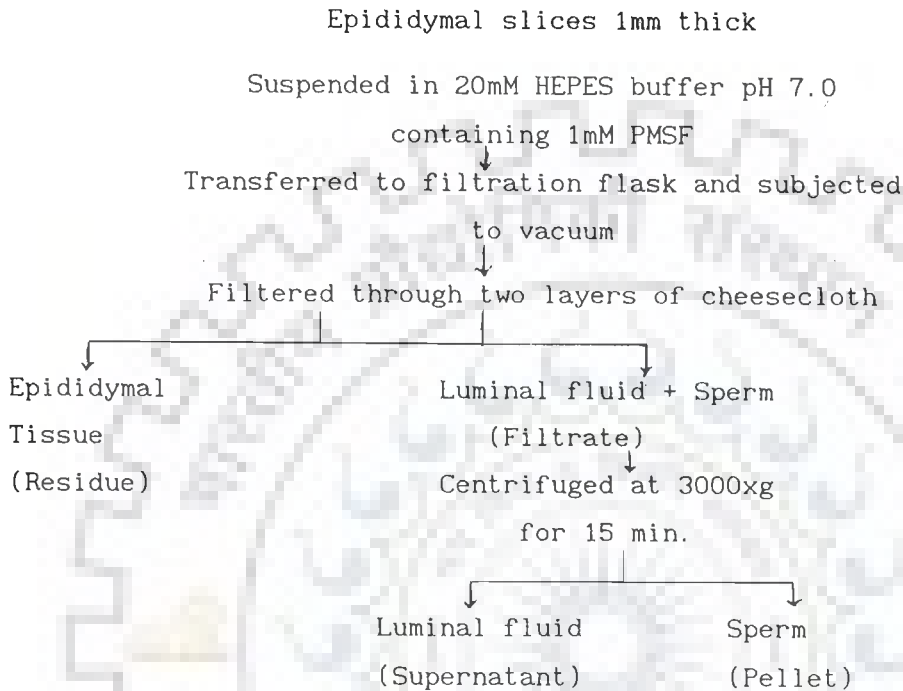
3.2.2   Preparation of Sperm Plasma Membrane

The method of Brooks, 1985, is used for the preparation of plasma
membrane from epididymal sperm, Sperm free from contaminating
epididymal epithelial cells, are first obtained by the procedure
just described.   The sperm thus obtained are washed in 20mM HEPES
(pH 7.0) buffer twice.   The sperm sample is sonicated on ice in a
Branson sonicator for 15 seconds at 100 watts in 10mM HEPES buffer
(pH 7.0), containing 1mM PMSF.   The sperm membranes released on
sonication are purified by differential and successive
centrifugation steps to obtain purified plasma membrane residues.
The exact procedure used for purification is given below in the
flow chart.

Fig. 3.2   Purification of Sperm Plasma Membranes

Epididymal sperm suspended in 10mM HEPES (pH 7.0)

containing 1mM PMSF.

Sonicated on ice for 15 seconds at 100W on a

Branson sonicator

Homogenate centrifuged at 6000xg for 20 minutes

Supernatant   Pellet

Centrifuged at 100,000xg/1 hour
on a ultracentrifuge

Pellet   Supernatant

Resuspended in 1.6 M sucrose by

gentle homogenization

The sample is transferred to the

bottom of the tubes

The sample is overlayed with 4ml

of 1.32M sucrose followed by 1M

sucrose

Centrifuged at 35,000xg for 90 minutes

on a Kontron Ultracentrifuge

(TFT70.35 rotor)

The plasma membrane recovered from

the 1.0M/1.32M sucrose interface

Membrane fraction is diluted with

10mM HEPES, pH 7.0 containing 1mM PMSF

Centrifuged at 100,000xg for 1 hour

Supernatant   Pellet contains purified

plasma membrane

3.2.3 Electron Microscopic Study by Negative Staining:

The sperm plasma membranes obtained were examined with an electron microscope.   Negative staining is used for staining the pure membrane preparations, as it is expected to give information about

(a)   structure of membrane

(b)   identity of membrane

(c)   damage suffered by membrane

Principle

The specimen is surrounded by an aqueous solution of an electron dense salt (the negative stain).   This is allowed to dry in a thin film, which is in effect an amorphous electron dense glass, in which the specimen is embedded.   The negative stain penetrates more or less into the hydrophilic parts of the specimen, and the resulting   image   in   the   electron   microscope   reveals   the unpenetrated regions as relatively electron transparent structures against an electron dense background.

The properties that characterize a useful negative stain are high solubility in water, high weight density and lack of interaction with the particular specimen under study.   Sodium and potassium phosphotungstate, uranyl acetate and ammonium molybdate have been most widely used as negative stains.   Uranyl formate, sodium tungstosilicate and tungstoborate have also been used.

Procedure

(a) Preparation of negative stain

Sodium phosphotungstate (5%): 2.5g phosphotungstic acid dissolved

in 30ml glass distilled water. The pH of the solution is adjusted to pH 7.0 with 1N KOH. The volume of the solution is made to 50ml.

(b) Preparation of carbon support film

A one inch square piece of mica sheet is cleaved with a sharp razor blade and used as the target for carbon coating. Two carbon electrodes are positioned on the brush holders such that the pointed electrode is spring held and tension is generated between it and the squared off electrode. Next, a clean white porcelain dish with a drop of vacuum oil on it is placed beneath the electrodes. The freshly cleaved mica is placed in the bell jar 10-15cm beneath the electrodes.

The bell jar is evacuated to $\sim 10^{-4}$ Torr. The carbon electrode is evaporated at an alternating current of $\sim$ 30mA at 15 V until the porcelain surrounding the drop turns light brown. The time taken in the process is about 30 to 45 seconds. Under these conditions the film thickness is approximately 70°A. The vacuum is released slowly. The mica coated with carbon film is lifted gently and the edge scraped with a razor blade very carefully. The mica is lowered slowly onto the surface of distilled water in a petridish at an angle $\leq 15^{o}$. Clean copper grids are lowered into the water beneath the carbon film without disturbing the film. The grids are then pulled through the film and blotted, surface side up on filter paper.

(c) Application of sample and staining

The carbon coated grids are placed surface side up on a clean glass slide. 10µl of the sperm suspension or membrane preparation is applied to the grid surface. After about 2 minutes, the excess sample is swabbed by touching the side of the grid with tissue paper. In a similar manner 10µl of 2% phosphotungstic acid is applied and 30 seconds later the excess stain is removed. The grid thus prepared is dried under a table lamp and viewed on a Phillips Transmission Electron Microscope at 80 kV.

3.2.4 Estimation of Adenyl Cyclase (White and Zenser, 1971)

Adenyl cyclase is a plasma membrane marker and the detection of adenyl cyclase in membrane preparations is an indication of purity of plasma membrane preparations. Determination of the activity of adenyl cyclase is usually performed with the use of a radioactive substrate, and requires purification from the reaction mixture of a radioactive product. This purification is a difficult one for two reasons:

1) The amount of product formed is several orders of magnitude less than the amount of precursor, and therefore, only a small contamination of product with precursor introduces a serious error.

2) The radioactive substrates are often themselves contaminated with small amounts of unknown radioactive impurities, which behave like 3',5'-cyclic nucleotides in certain purification systems. 3',5'-cyclic nucleotides are purified by chromatography on columns of aluminium oxide.

The adenyl cyclase assay is carried out in 10x75mm tubes and incubated at $30^{o}C$. Final volume of the reaction mixture in assay is 0.075ml which contained 50mM Tris-HCl buffer (pH 7.6), 0.1mg bovine serum albumin and 20mM caffeine. The adenyl cyclase reaction mixture contains 1.2mM (α -^{32}P) ATP, approximately 5×10^5 cpm, 5.0mM $MgCl_2$, 15μg creatine phosphokinase, 25mM creatine phosphate, and 0.2μM 3',5'-AMP. The reaction is initiated by adding 0.025ml of the membrane preparation and, after incubation with shaking for 10 min, reaction is terminated by adding 0.02ml of 0.1M sodium ethylenediaminetetraacetate (pH 7.6), containing approximately 30,000 cpm of (^{3}H) 3',5'-AMP, 2.35 Ci/mM. The tubes are then heated to $100^{o}C$ for 3 min in an electric heating block and cooled in an ice bath. To each tube is added 1.0 ml of 0.05M Tris-HCl buffer, (pH 7.6), with an automatic pipette. The contents are stirred on a vortex mixer and the tubes centrifuged for 10 min at 2500xg. The supernatant from each tube is applied to an alumina column, and the proper column fraction collected and counted for ^{3}H and ^{32}P. Losses of (^{32}P) cyclic nucleotide are corrected for from the amount of (^{3}H) 3',5'-AMP recovered.

Approximately 1.0gm of neutral alumina is measured out and is wetted with 0.05M Tris-HCl buffer (pH 7.6) and washed into a column with about 15ml of the same buffer dispensed from a wash bottle. The effluent pH is monitored for the particular brand of alumina used to be sure it reads 7.6. The columns are washed extensively with buffer. The columns are then allowed to drain.

The sample to be chromatographed, contained in 1.0 ml of the above buffer, is applied to a prepared column of alumina with a disposable pasteur pipette. At pH 7.6, about 80% of applied 3',5'-AMP appears in the next 2 ml eluting from the column. Thus, after the application volume is drained to recover 3',5'-AMP. The rack of columns is lifted from the draining box and placed onto the rack of scintillation vials. Then 2ml of Tris-HCl buffer is applied to each column and this is allowed to drain into the vials.

After removal of the columns, to each vial is added 10ml of Bray scintillation mixture and the radioactivity is measured in a liquid scintillation spectrometer, Beckman LS 1202.

3.2.5 Biochemical Analysis of Sperm Plasma Membrane

Several protocols are currently available for the simultaneous analysis of a number of membrane associated parameters. One such procedure has been described by Glossman and Neville (1971) for the analysis of plasma membranes from rat liver, kidney brush border and erythrocyte ghost cells. The methodology described by them has been adapted in the present study for the simultaneous analysis of sperm membrane parameters.

Briefly, sperm membranes from the twelve different segments are precipitated with 1% phosphotungstic acid in 0.5N HCl. The precipitate is then washed twice with 5% Trichloroacetic acid at room temperature and pelleted by centrifugation at 5000xg for 20 minutes on a table top centrifuge. The pellet is dried in a

Fig. 3.3 Biochemical Analysis of Sperm Plasma Membrane

Sperm plasma membrane

Precipitate with 1% Phosphotungstic acid
in 0.5N HCl, wash twice with 5% TCA

Centrifuge

→ Supernatant

Pellet dried at
room temperature in desicator

Dissolve Pellet
in 0.1N NaOH

Hydrolyse in 0.1N
H_2SO_4 at 85°C

Delipidation by
suspending in 0.5ml
methanol

Protein analysis
(Lowry et al., 1951)

Sialic Acid analysis
(Warren, 1959)

Add 1ml of chloro-
form and incubate
suspension for 2 hrs
at 45°C.

Centrifuge for 4 min
at 12,000xg. Repeat
twice and pool

Pellet

Supernatant

Neutral Sugars
(Roe, 1955)

Hydrolyse with
2N HCl at 100°C
for 16 hrs.

Dry

Dry

Cholesterol
(Hanel and Dam,
1955)

Digest with 10%
$MgNO_3$ in Ethanol

for 1hr at 200°C

Amino Sugar Analysis
(Gatt and Berman, 1966)

Inorganic Phosphate
(Fiske and Subbarow,
1925)

lyophilizer. The dried pellet is weighed and divided into three parts for various biochemical analyses. One part of the pellet is dissolved in 0.1N NaOH and suitable aliquots of this solution are used to quantitate protein by the method of Lowry et al., (1951).

The second part of the pellet is hydrolysed in 0.1N Sulfuric acid for one hour at 80°C. The hydrolysate is used for the estimation of sialic acid by the Thiobarbituric acid assay method of Warren (1959).

The third part of the pellet is subjected to delipidation by the following method. The membrane pellet is suspended in 0.5ml methanol followed by 1.0ml of chloroform. The suspension is incubated for 2 hours at 45°C. The suspension is centrifuged at 12,000xg for 4 min on a Beckman J2 21 centrifuge. The process is repeated twice and the supernatants are pooled.

The supernatant now available is divided into two parts. One part is used for the estimation of cholesterol (Hanel and Dam, 1955). The solution is first dried and reconstituted in chloroform for this purpose. The second portion of the supernatant is dried and digested with 10% magnesium nitrate in ethanol. The amount of phospholipids present is determined by estimating the inorganic phosphate content of the digest by the method of Fiske and Subbarow (1925).

The delipidated pellet is divided into two parts, one part is used for the estimation of neutral sugars by the anthrone method of Roe (1955). The second part of the pellet is hydrolysed with 2N HCl

for 16 hours at $100^{O}C$ and the amino sugar content of the hydrolysate estimated by the method of Gatt and and Berman (1966).

The consolidated scheme for estimation of the biochemical parameters is given in Fig. 3.3 in the form of a flow chart.

3.2.5.1 Statistical analysis

The significance of the changes in biochemical components on sperm surface during epididymal transit are analysed by the one way analysis of variance using F-distribution (Spiegel, 1982).

3.2.5.2 Estimation of protein

Protein content of the membrane pellets is estimated by the method of Lowry et al., (1951). The final color developed is a result of:

(i) Biuret reaction of protein with copper ions in alkali.

(ii) Reduction of the phosphotungstic phosphomolybdic reagent with tyrosine and tryptophan present in the treated protein.

Reagents

Reagent A: 2% Sodium carbonate in 0.1N Sodium hydroxide.

Reagent B: 0.5% Copper sulfate pentahydrate in 1% Potassium sodium tartrate. Prepared fresh before use.

Reagent C: Alkaline copper reagent comprising 50 parts of Reagent A and 1 part of Reagent B.

Reagent D: Folin Ciocalteau reagent made 1N in acid.

Protein Standard: A 20-200μg Bovine serum albumin/ml diluted from a stock solution of 1mg/ml Bovine serum albumin.

Procedure

1. Aliquots containing suitable amounts of protein are made upto 1ml in 0.1N Sodium hdroxide.

2. To this 1ml of Reagent C is added and mixed.

3. The mixture is then incubated for 15 minutes at room temperature.

4. 0.1ml of Reagent D is added and mixed vigorously immediately.

5. The absorbance at 690nm is recorded on a Beckman DU-6 spectrophotometer after 45 minutes incubation at room temperature, using a reagent blank.

3.2.5.3 Estimation of sialic acids

Sialic acids form β-formylpyruvic acid upon oxidation with periodate. It is well known that the thiobarbituric acid chromophores of sialic acids and 2-keto-3-deoxygluconic acid behave similarly. The chromophore is soluble in cyclohexanone and slightly soluble in isoamyl alcohol and both are unstable in basic solutions in contrast to malonaldehyde chromophore. The Thiobarbituric acid assay method of Warren (1959) is a rapid and sensitive method for the estimation of only free sialic acids. Therefore, known amounts of precipitated and dried sperm membrane are hydrolysed in 0.1N H_2SO_4 for 1 hour at $80^{\circ}C$. This procedure releases the bound sialic acid from sperm membranes. Thus, assay of sialic acids in the hydrolysate is a measure of total sialic acids (bound + free).

Reagents

Reagent A: 0.2M Sodium metaperiodate in 9M Phosphoric acid

Reagent B: 10% Sodium arsenite in 0.5M Sodium sulfate in 0.1N Sulfuric acid.

Reagent C: 0.6% Thiobarbituric acid in 0.5N Sodium sulfate

Reagent D: Cyclohexanone.

Procedure

1. To 0.2ml acid hydrolysate, 0.1ml Sodium metaperiodate solution is added and mixed.

2. The tubes are incubated at room temperature for 20 minutes.

3. 1.0ml Sodium arsenite solution is added and the tubes shaken till the yellow brown precipitate disappears.

4. 3.0ml of Thiobarbituric acid solution is added to this.

5. The tubes are capped with glass beads and placed in a vigorously boiling water bath for 15 minutes. Thereafter, tubes are removed and cooled in water for 5 minutes.

6. 4.3ml of cyclohexanone is added and mixed to this solution so that the chromophore is transferred from the aqueous phase to the organic phase.

7. This mixture is centrifuged for 3 minutes on a table top centrifuge.

8. The optical density of the clear upper organic layer is recorded at 532 and 549nm on a Beckman DU-6 spectrophotometer.

The sialic acid content of the test solution is determined from

the following equation

$$\text{mmoles of N-acetylneuraminic acid} = 0.090 \times OD_{549} - 0.033 \times OD_{532}$$

This equation corrects the optical density at 549nm not derived from N-acetylneuraminic acid. The correction is based on the observed molecular extinction values of N-acetylneuraminic acid and 2-deoxyribose at 532 and 549nm respectively.

3.2.5.4 Estimation of cholesterol

This is a simple and sensitive method for the determination of cholesterol based on Tschugaeff's color reaction.

Reagents

Reagent A: Chloroform.

Reagent B: 40g Zinc chloride in 153ml Glacial acetic acid.

Reagent C: 98% Acetyl chloride.

Standard : A working stock solution of 100µg/ml Cholesterol in Chloroform.

Procedure

1. To each tube containing samples in 2ml Chloroform, 1ml of the Zinc chloride reagent is added.

2. To this 1ml of Acetyl chloride is added. Acetyl chloride is most conveniently dispensed from a burette.

3. The contents of the tube are then mixed with a glass rod, and tubes are placed in a water bath at $60^{o}C$ for 10 minutes.

4. The absorbance is recorded at 528nm on a Beckman DU-6 spectrophotometer using a reagent blank.

3.2.5.5 Estimation of total phospholipid

The phosphorus content of sperm membrane preparations is quantified by the method of Fiske and Subbarow (1925). The organic phosphorus compounds are oxidized to orthophosphates in the presence of perchloric acid. The orthophosphates form color complexes with molybdate in the presence of Amino naphthol sulfonic acid reagent. The value obtained for phosphorus in this procedure is multiplied by 25 to give an estimate of total phospholipids (Beiri and Prival, 1965).

Reagents

Reagent A: 72% Perchloric acid

Reagent B: 0.0405M Ammonium molybdate

Reagent C: 1.15M Sodium bisulfite; 0.191M Sodium metabisulfite, and 0.081M 1,2,4, Amino naphthol sulfonic acid

Standard : 1μmole/ml Potassium dihydrogen phosphate

Procedure

1. Suitable lipid aliquots are evaporated to dryness on a sand bath.

2. 0.8ml perchloric acid is added and the samples are digested for 20 minutes on the sand bath.

3. Samples cooled to room temperature.

4. 6.0ml water, 0.5ml Reagent B and 0.4ml Reagent C are added and mixed well. The final volume is made upto 10ml with water and mixed.

5. The samples are incubated for 20 minutes at room temperature.

6. Absorbance is recorded at 660nm on a Beckman DU-6

spectrophotometer using reagent blank and 1μmole/ml potassium dihydrogen phosphate is run as a standard simultaneously.

3.2.5.6 Estimation of neutral sugars

This estimation is done by the method of Roe (1955) using Anthrone reagent and is based on the formation of furfural derivatives in concentrated sulfuric acid which react with anthrone to form a blue green chromophore. The method is applicable to glycoproteins directly without prior hydrolysis. Hexosamines do not interfere in this reaction, though sialic acids yield a chromophore equivalent to 8% of the colour given by galactose on a molar basis.

Reagents

Reagent A: 280ml of chilled water is slowly added to 720ml concentrated Sulfuric acid. The mixture is cooled and 500mg Anthrone is added to it. The reagent is mixed and allowed to stand for at least four hours before use.

Standard : 20μg/ml working stock solution of glucose in distilled water.

Procedure

1. To samples containing upto 20μg Neutral sugars made upto 1ml in distilled water, 5ml Reagent A is added and mixed.

2. Tubes are capped with marble and incubated for 15 minutes in a boiling water bath.

3. The tubes are cooled to room temperature and absorbance recorded at 620nm using a reagent blank.

3.2.5.7 Estimation of amino sugars

The estimation of amino sugars has been done by the method of Gatt and Berman (1965).   The method is a modification of the original method given by Elson and Morgan (1933).   Amino sugars on heating in alkaline solution with Acetyl acetone are converted into pyrole derivative which on treatment with p-dimethyl aminobenzaldehyde gives rise to a stable pink chromogen.   The sperm membrane amino sugar content has been estimated by first hydrolysing the samples in 0.6ml of 2N HCl at 100°C for 16 hours in a screw cap tube, sealed and stoppered tightly.

Reagents

Reagent A: 2N Hydrochloric acid

Reagent B: 2M Sodium carbonate

Reagent C: 2% Acetyl acetone in 1.5M Sodium carbonate

Reagent D: Absolute Alcohol

Reagent E: Ehrlich's reagent: 1g p-Dimethyl aminobenzaldehyde in a mixture of 15ml Ethanol and 15ml concentrated Hydrochloric acid

Standard : Working stock solution of 20μg/ml Galactosamine hydrochloride in 2N HCl

Procedure

1.   To samples containing 1-20 μg amino sugars in 0.6ml, 0.4ml Reagent B is added and mixed gently.

2.   0.5ml of 2% Acetylacetone is then added to the tube.

3.   The tubes are stoppered tightly and placed in boiling water bath for 20 minutes.

4.   The tubes are cooled and 1.0ml Ethanol is added.

5.   To this 0.5ml Reagent E is added and mixed vigorously.

6.   The absorbance is recorded at 530nm after five minutes using a reagent blank.

3.3 RESULTS

Isolation of sperm from different segments of the epididymis free from contamination of the cells lining the epididymal epithelium is a prerequisite for the current study. The concentration of sperm extracted from the three regions of the epididymis, the caput, corpus and cauda, at (a) varying pressures of evacuation at a fixed time of evcauation (Fig. 3.4) and (b) varying periods of evacuation and fixed evacuation pressure (Fig. 3.5). From the data presented, it is apparent that an evacuation pressure of 200 mm Hg and evacuation time of 2 minutes are most conducive for obtaining uncontaminated sperm preparations. At higher pressure (>200 mm Hg) and longer time of evacuation (>2 minutes) result in the contamination of sperm suspension by epididymal epithelial cells. While at low evacuation pressure (<200 mm Hg) and shorter evacuation times the yield is lower. The parameters for pressure and time of evacuation standardized for the three epididymal regions have been used for the removal of sperm from the twelve epididymal segments.

Preparation of sperm plasma membrane from the twelve epididymal segments is achieved using the method of Brooks (1985). The sonicated sperm samples subjected to sequential centrifugation steps give the purified membrane preparations at the 1.0/1.32M sucrose gradient interface.

The purity of the membrane preparations has been assessed by the (a) electron microscopic analysis of the membrane vesicles (Fig. 3.6), and (b) detection of adenyl cyclase activity (Fig. 3.7). From the electron microscopic study it is apparent that the round vesicles visible after negative staining by phosphotungstic acid are purified membrane vesicles. The lack of contamination by cellular debris is also evident.

The adenyl cyclase activity of the purified membrane vesicles falls between 36.51 ± 1.58 pmol of cAMP liberated per minute per mg protein in the first segment to 27.09 ± 2.19 pmol of cAMP liberated per minute per mg protein in the twelth segment. Adenyl cyclase is a plasma membrane marker and the detection of adenyl cyclase activity in the membrane preparations confirms the presence of sperm plasma membrane.

The present study highlights the changes in lipids, carbohydrates and protein composition of sperm plasma membrane purified from twelve segments of the epididymis of goat.

The quantification of the lipid components (phospholipids and cholesterols) in the membrane preparations of the sperm from 12 segments of the epididymis is given in Fig. 3.8 and Fig. 3.9. There is a significant decrease in the levels of phospholipids and cholesterol ($P<0.05$) as the sperm move from the first segment to the twelth segment. The level of phospholipid in the first segment, estimated at 9.618 ± 0.104 μg/mg sperm membrane, fell to 5.190 ± 0.018 μg/mg sperm membrane in the twelth segment. The

loss of sperm membrane phospholipid is most rapid in the first five segments, while thereafter the reduction is rather gradual. In the first five segments the loss in phospholipids amounts to 30%, while in the succeeding segments it is merely 16%. The loss of sperm surface cholesterol in the twelve segments is uniform over all the segments of the epididymis. The total cholesterol content diminished from 3.991 ± 0.657 μg/mg sperm membrane in the first segment to 2.686 ± 0.079 μg/mg sperm membrane in the twelth segment, a loss of 32%. The level of cholesterol is considerably lower than the level of phospholipids in the epididymal spermatozoa. The loss of phospholipids in the epididymis is more pronounced as compared to the depletion of cholesterol. This difference in the extent of removal of phospholipids and cholesterol in sperm from different segments is reflected in cholesterol to phospholipid ratios. In contrast to the fact that both the cholesterol and phospholipids are lost during epididymal transit, the cholesterol to phospholipid ratio increases from 0.41 to 0.51 from the first to twelth segment (Fig. 3.10).

The composition of carbohydrates on sperm membrane in terms of neutral sugars, amino sugars and sialic acids is given in Fig. 3.11, Fig. 3.12 and Fig. 3.13 respectively. The sperm membrane neutral sugar content falls from 25.405 ± 0.172 μg/mg sperm membrane in the first segment to 13.074 ± 0.665 μg/mg sperm membrane in the twelth segment. The change in neutral sugar is significant at $P < 0.05$ and the loss amounts to 48%. It is apparent that the loss of neutral sugars is rapid in the first

five segments where a total of 32.6% of the neutral sugars are removed from the sperm membrane. In contrast, 16% of the neutral sugars are removed in the succeeding segments.

There is a significant reduction in the level of amino sugars during epididymal transit ($P < 0.05$). The level of amino sugars in the first segment is estimated at 5.733 ± 0.253 µg/mg sperm membrane, while in the 12th segment the level is quantitified at 3.249 ± 0.033 µg/mg sperm membrane, a loss of about 43%. Unlike the rapid depletion in the level of neutral sugars in the first five segments, the reduction in amino sugar content of the epididymal spermatozoa is gradual over the entire course of the epididymis.

The sialic acid composition of sperm membrane preparations undergoes a drastic reduction during epididymal transit and the change is significant at $P < 0.01$. The composition of sperm surface sialic acid in the first segment is 4.79 ± 0.543 µm/mg sperm membrane and the level is reduced by about 80% to 0.9265 ± 0.138 µm/mg sperm membrane in the twelth segment. The depletion in sperm membrane sialic acid in the first five segments of the epididymis is rapid with a loss of 62%. In the succeeding segments the loss of sperm surface sialic acid, in contrast amounts to mere 18%. Thus, only 20% of the sialic acid present on the sperm surface in the first segment survives during epididymal transit.

Fig. 3.4   Effect of Variation in the Time of Evacuation on the
Isolation of Sperm from the Three Segments of the
Epididymis (▨▨▨) Caput, (▤▤▤) Corpus and (▥▥▥)
Cauda at 100 mmHg. Evacuation was carried out for (A)
0.5 min, (B) 1.0 min, (C) 1.5 min, (D) 2.0 min and (E)
3.0 min. The Sperm count (per $mm^3 \times 10^5$) and Total cell
(per $mm^3 \times 10^2$) are expressed as Mean ± SEM of five
observations.

Fig. 3.5   Effect of Variation in the Evacuation Pressure   on
Isolation of Sperm from the Three Segments of the
Epididymis (▨▨) Caput, (▤▤▤) Corpus and (▥▥▥)
Cauda. The evacuation pressure applied was (A) 50 mmHg,
(B) 100 mmHg, (C) 200 mmHg, (D) 250 mmHg (E) 300 mmHg
for 2 min. The Sperm count (per $mm^3 \times 10^5$) and Total cell
count (per $mm^3 \times 10^2$) are expressed as Mean ± SEM of five
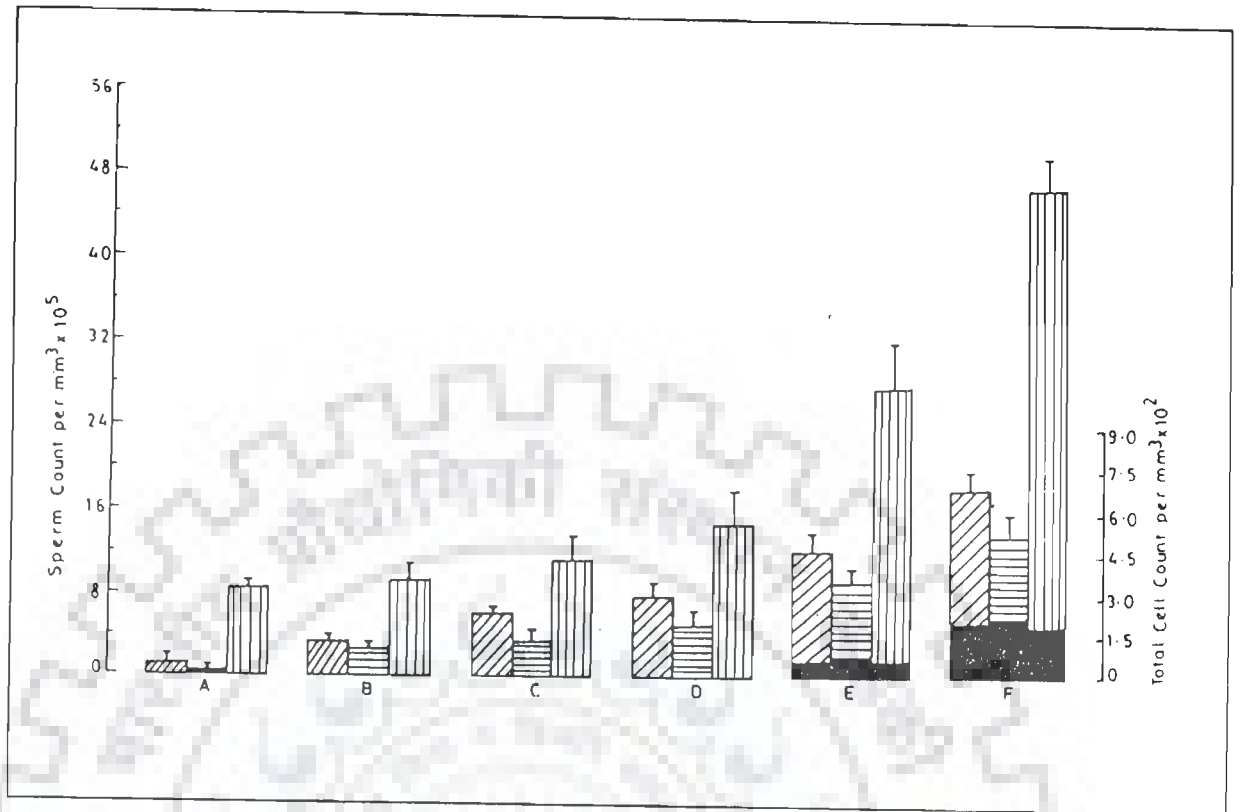observations.

FIG. 3·4

71·285 ± 4·86
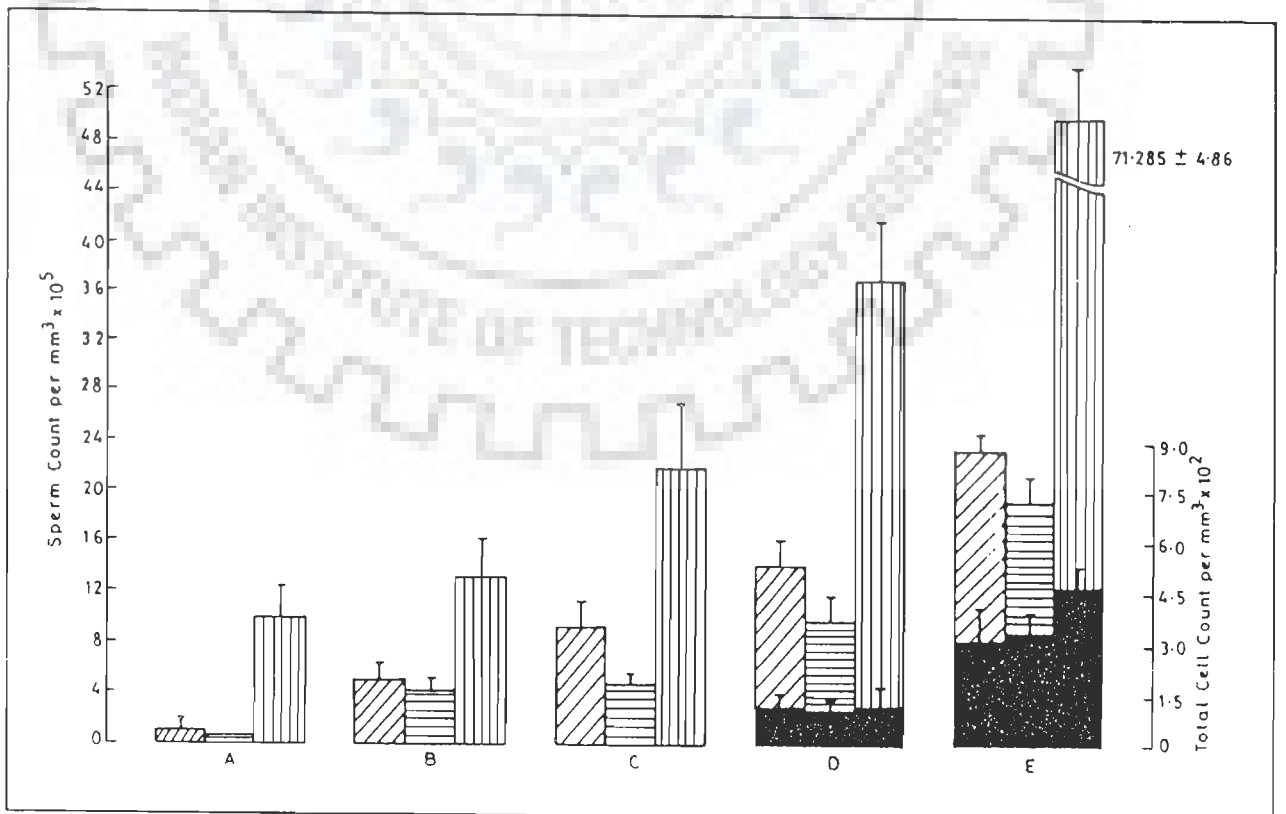
FIG. 3·5

Fig. 3.6(a) Different Segments of the Goat Epididymis (Besancon et al., 1985).

(b) Electron Micrograph of goat epididymal spermatozoa (X1920) after negative staining.

(c) Electron Micrograph of Sonicated sperm sample (X2400) after negative staining.

(d) Electron Micrograph of Sperm plasma membrane vesicles (X84000) after negative staining.

(a)

(b)

(c)

FIG. 3·6

(d)
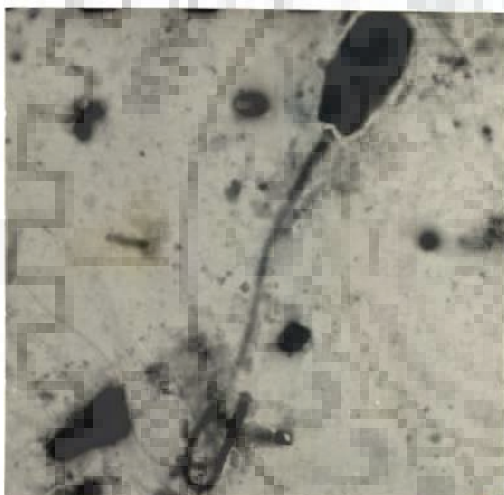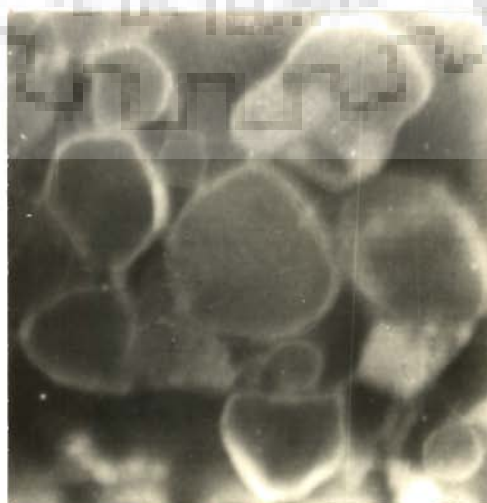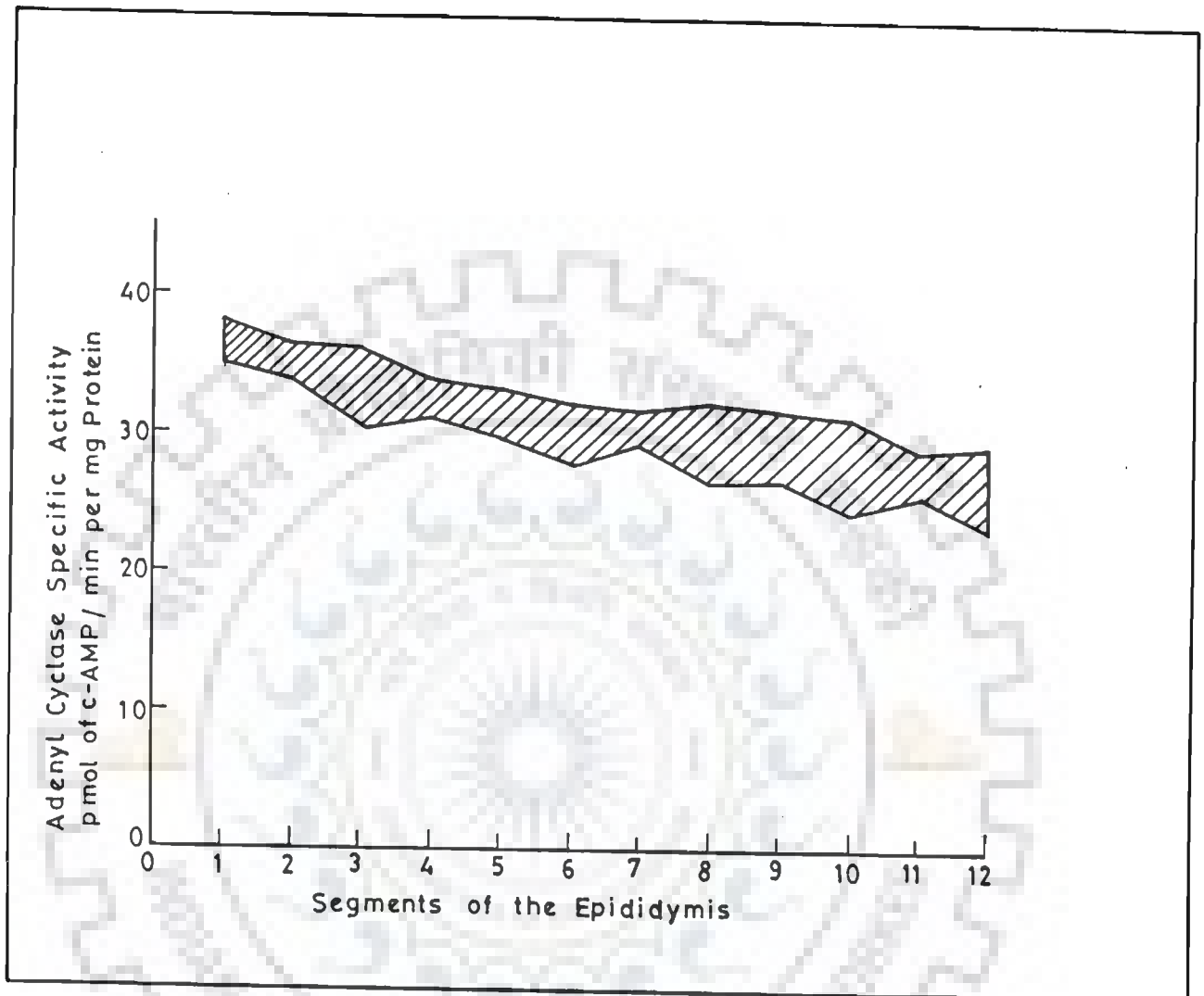
Fig. 3.7 Distribution of Adenyl Cyclase Activity in the Sperm
Plasma Membrane Preparations for the Twelve Segments of
the Epididymis expressed as pmol of c-AMP/min per mg
Protein. The shaded area represents the Standard
Deviations in enzyme activity of five observations in
the different segments.

64

Fig. 3.8   Distribution of Sperm Membrane Phospholipids (µg/mg
Sperm Membrane) in the Membrane Preparations from the
Twelve Segments of the Epididymis. The bars represent
Mean ± SEM of five observations.
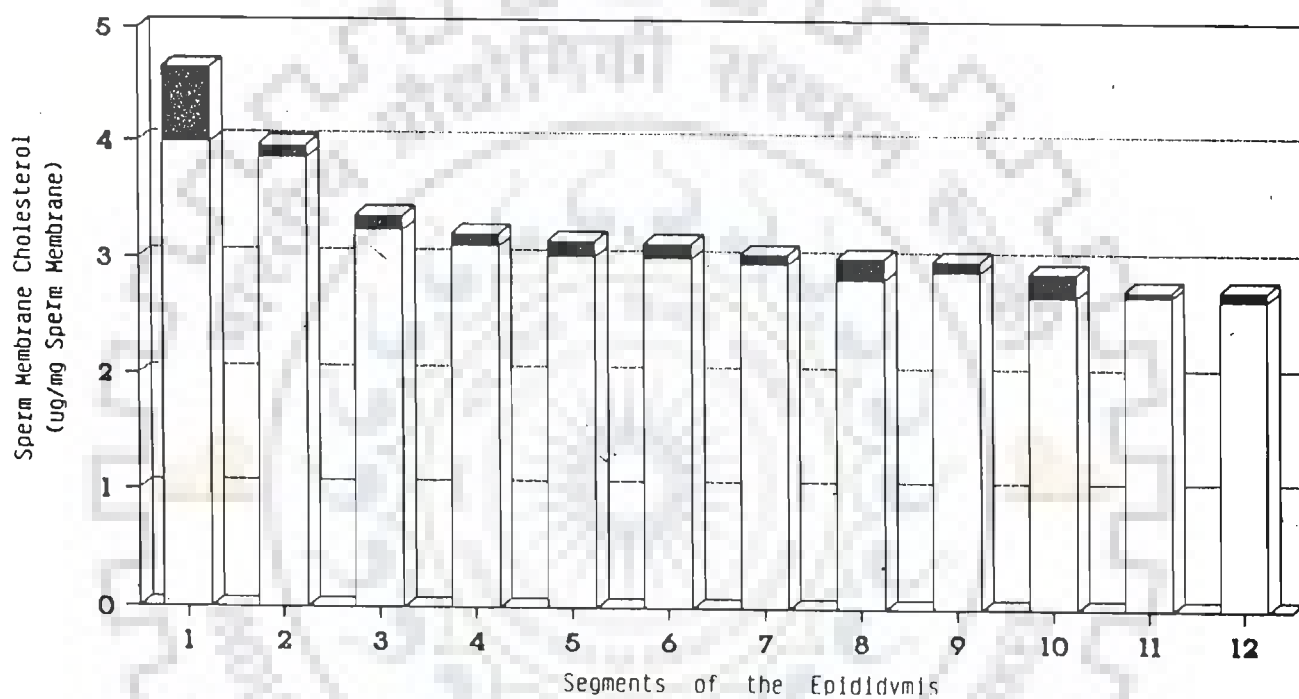
Fig. 3.9   Distribution of Sperm Membrane Cholesterol (μg/mg Sperm
            Membrane) in the Membrane Preparations from the Twelve
            Segments of the Epididymis.   The bars represent Mean ±
            SEM of five observations.
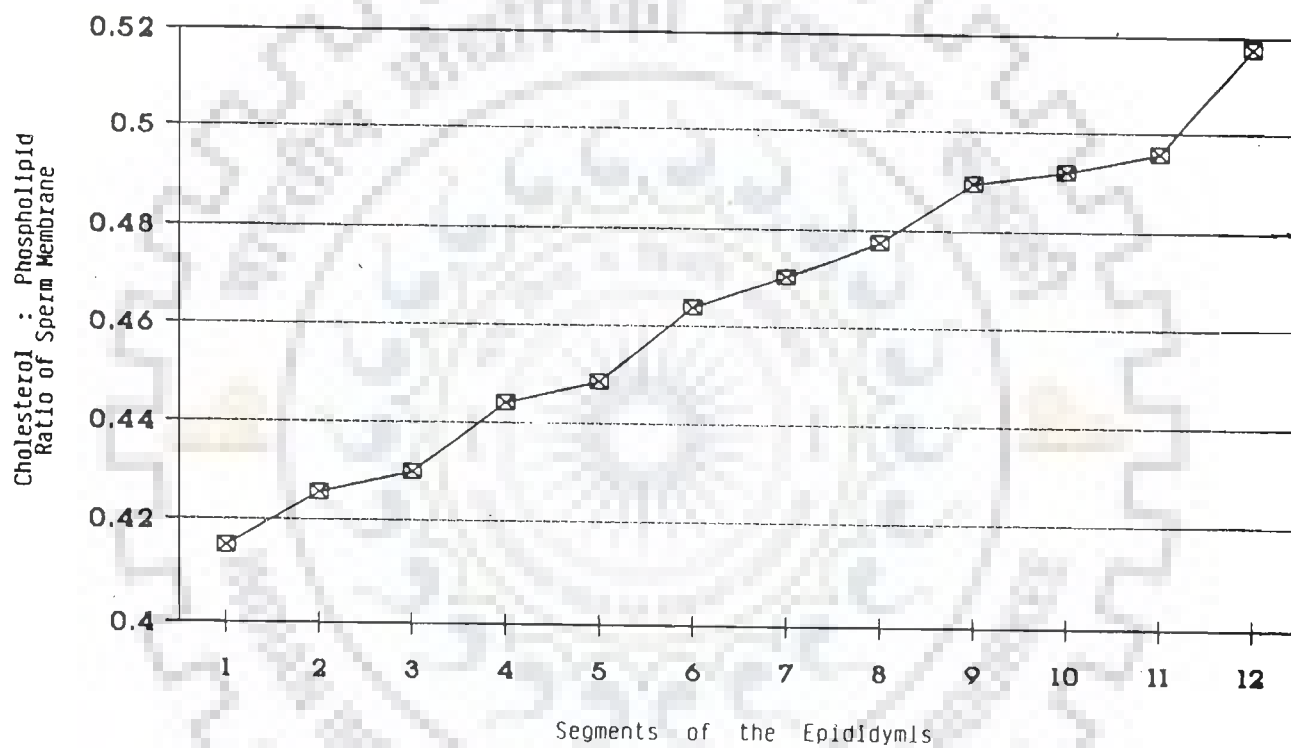
Fig. 3.10 The Cholesterol: Phospholipid Ratio of Sperm Preparations from the Twelve Segments of the Epididymis.
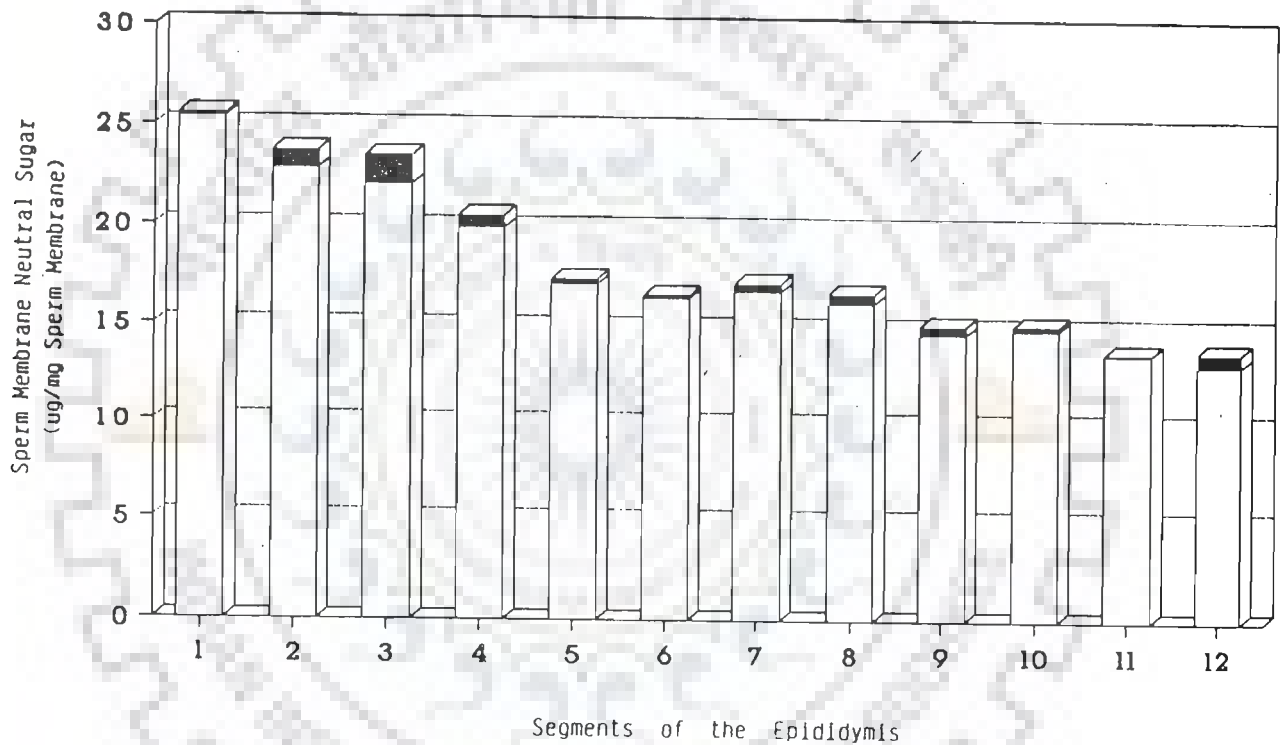
Fig. 3.11 Distribution of Sperm Mambrane Neutral Sugars (μg/mg Sperm Membrane) in the Membrane Preparations from **the Twelve Segments of the Epididymis.** The bars represent the Mean ± SEM of five observations.

Fig. 3.12 Distribution of Sperm Membrane Amino Sugars (μg/mg Sperm
Membrane) in the Membrane Preparations from the Twelve
Segments of the Epididymis. The bars represent the mean
± SEM of five observations.

Fig. 3.13 Distribution of Sperm Membrane Sialic Acids (μM/mg Sperm Membrane) in the Membrane Preparations from the Twelve Segments of the Epididymis. The bars represent the mean $\pm$ SEM of five observations.
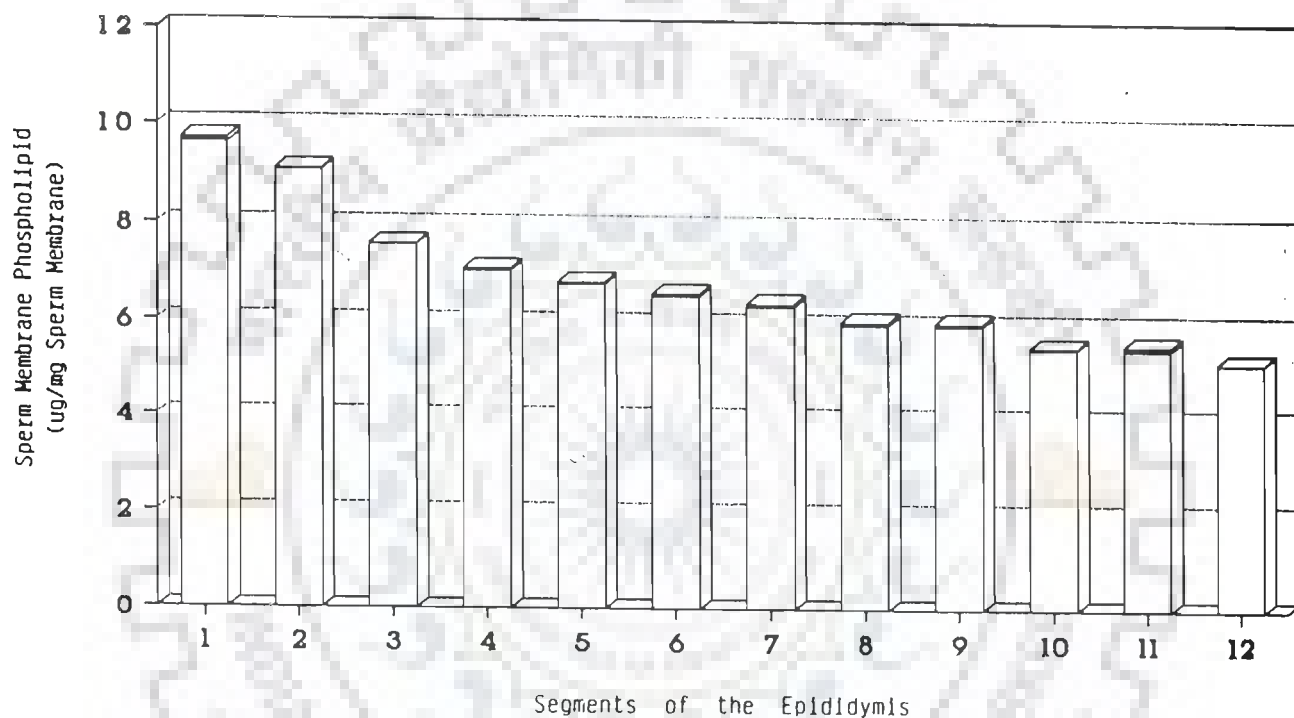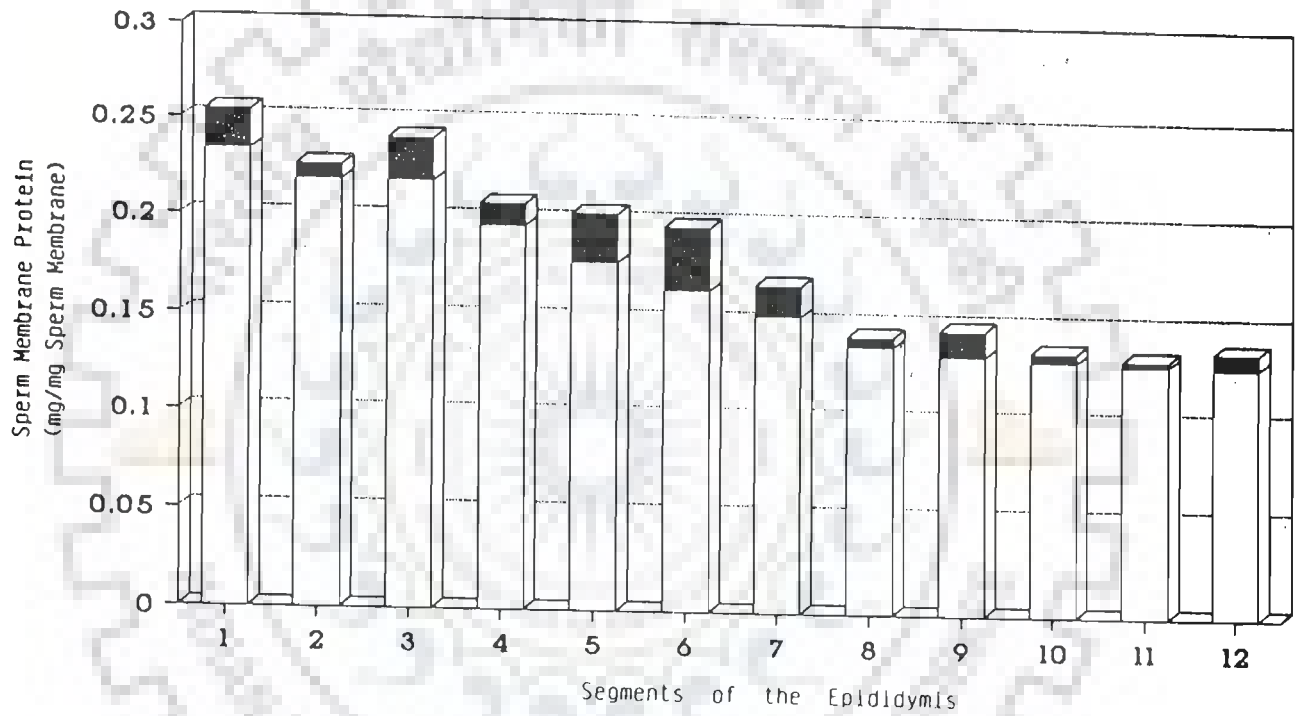
Fig. 3.14 Distribution of Sperm Membrane Protein (mg/mg Sperm Membrane) in the Membrane Preparations from the Twelve Segments of the Epididymis. The bars represent the Mean ± SEM of five observations.
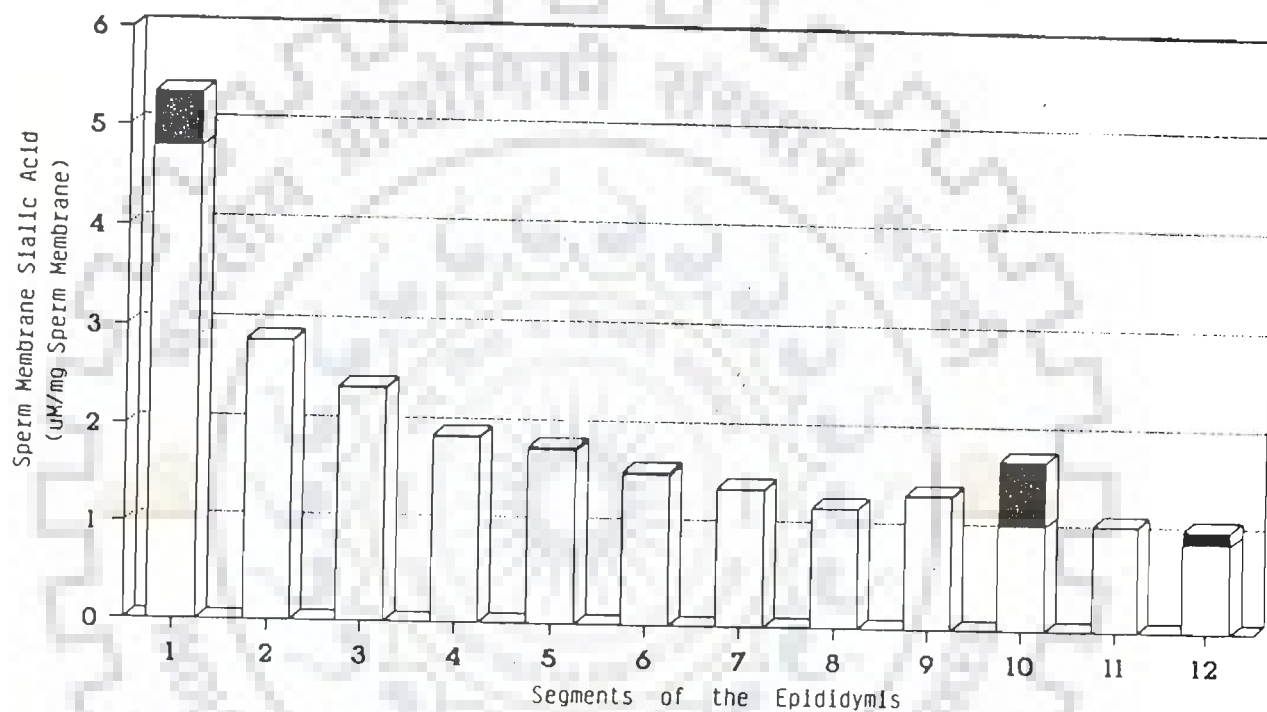
Of the three carbohydrate classes quantified on sperm membrane preparations from the twelve segments of the epididymis, the neutral sugars constitute the major portion followed by, amino sugars and finally sialic acids.

The depletion in the protein content of the sperm membrane from the sequential segments of the epididymis is significant ($P < 0.05$). From Fig. 3.14 it is apparent that the level of protein in the sperm membrane of the first segment is 0.234 ± 0.02 mg/mg sperm membrane, while the level goes down to 0.128 ± 0.008 mg/mg sperm membrane in the twelth segment. The depletion in protein content of epididymal sperm membrane preparation is 41% in the first eight segments. There is a stabilization in the sperm surface protein content in the succeeding four segments, with a loss of just 3% recorded in these segments. Proteins constitute a bulk of the sperm surface components studied here.

3.4 DISCUSSION

Previously, attempts have been made to recover sperm from epididymal tissue slices by gentle agitation after suspending them in known volumes of desired buffers (Hall and Killian, 1987). Although the procedure has not been critically reviewed, from personal experience it is apparent that there is a need for further purification to obtain sperm free from epithelial cells. The procedure adapted in the present study is the first of its kind that employs vacuum to liberate sperm from epididymal tissue slices. By this procedure too, there is a concern of possible contamination from the epididymal epithelial cells in the extrudate, but the data obtained suggests that this can be delicately controlled. At low vacuum there is virtually no

contamination from epididymal epithelial cells, but the yield in terms of intact sperm obtained is also low. On the other hand, the use of higher vacuum increases both the number of sperm and epithelial cells liberated, a feature which is not desirable. The contamination from the epididymal epithelial cells increases with the time of exposure to vacuum. Thus, the intensity of evacuation pressure used and the time of exposure to such an evacuation is very critical. It was found that exposure to evacuation pressure of 200 mm Hg for 2 min, is optimal for obtaining pure sperm preparations from virtually any segment of the epididymis.

A number of procedures are available for the preparation of sperm plasma membranes. Nitrogen cavitation (Peterson et al., 1980 and Noland et al., 1983) and other methods require specialized apparati. Although, membranes of high quality are obtained by these methods, not all investigators can afford to acquire these instruments. Simpler schemes, employing sonication of sperm and purification of its membranes by ultracentrifugation, are available (Brooks, 1985).

In the present study, the sonication of sperm yields good amount of sperm membrane vesicles which are purified by centrifugation on sucrose gradients. The quality of membrane obtained by this procedure appears to be excellent as evidenced by in a transmission electron microscopy.

A similar approach for assessing the purity of sperm membrane preparations by electron microscopy has been used in the past, but

with a slight variation in the staining procedures (Hall and Killian, 1989 and Tulsiani et al., 1990). The detection of adenyl cyclase activity confirms that the preparation is in fact that of sperm plasma membrane.

The acquisition of motility and fertilizing ability by sperm occurs in concert with quantitative changes in lipids, carbohydrates and proteins of sperm membranes observed during epididymal transit.

The data obtained in the present study clearly demonstrates that lipid composition of sperm membranes dramatically changes during epididymal maturation. Previous investigators have similarly shown that lipids are lost from the sperm of several animals during passage through the epididymal duct (Grogan et al., 1966; Quinn and White, 1967; Poulos et al., 1973ab, 1975; Evans and Setchell, 1979 and Parks and Hammerstedt, 1985). Phospholipids and cholesterol are recognized to be important constituents of sperm membrane (Jones and Mann, 1976). Quantitative analysis of both these components showed that the reduction in the level of phospholipid was more pronounced as compared to that of cholesterol. Phospholipases have been reported to be active in the epididymis (Terner et al., 1975; Llanos et al., 1982). In addition, it has been pointed out that sperm can utilize the fatty acid side chains of phospholipids as a source of metabolizable substrate for energy yielding pathways (Tang and Hoskins, 1974 and Evans and Setchell, 1978). This is likely one cause for depletion in sperm membrane phospholipid.

An important consequence to the disproportional reduction in the level of phospholipid and cholesterol is clearly reflected in the cholesterol: phospholipid ratio. The values obtained for sperm membrane changed from 0.41 to 0.51 during epididymal transit. Just as in the present study, similar observations have been made earlier (Parks and Hammerstedt, 1985 and Mohri et al., 1989). Boar sperm plasma membrane cholesterol: phospholipid ratio in contrast decreases from 0.16 in caput to 0.12 in caudal cells (Niklopoulou et al., 1985). Such a dramatic change in the ratio has far reaching implications. Membrane permeability, fluidity and rigidity are known to be governed by the level of phospholipids and cholesterol (Mann and Lutwack Mann, 1981; Bearer and Friend, 1982; Parks and Hammerstedt, 1985 and Shivaji and Jagannadham, 1992). Decrease in membrane fluidity may contribute to the maintenance of lipid domains by depressing the lateral diffusion rates of anionic lipids from adjacent regions (Bearer and Friend, 1980, 1981 and 1982). In sperm this might be one factor that maintains the identity of the five domains in the plasma membrane. It has been proposed that cholesterol/phospholipid ratio of sperm should reach a level ideal for acrosome reaction and capacitation to occur for subsequent interaction with the egg for fertilization (Davis, 1981). The reduction of phospholipids results in changes in sperm physiology related to heat resistance (Bishop, 1961 and Voglmayr et al., 1967), response to cold shock (Quinn and White, 1968a,b and Nikolopoulou et al., 1985) and resistance to harmful alkaloids (Tulsiani et al., 1990). Perhaps the change in the ratio occuring

in the sperm plasma membrane during epididymal transit is causally related to making this possible.

Until now most investigators have used affinity labeling of specific sugars as a means to monitor the qualitative changes in carbohydrate moieties of the sperm surface. The degree of binding is rather arbitrarily defined as weak, moderate and strong. Lectin binding studies were very useful since they could identify terminal sugars which may be exposed by unmasking or disappear due to masking. Thus, important clues could be obtained regarding the type of sugar moieties involved in events leading to fertilization. Very few reports, however, deal with the quantitative changes in sperm surface carbohydrate moieties.

In the present study both neutral sugars and amino sugars were measured separately in plasma membrane of sperm collected from twelve segments of the goat epididymis. Sperm membranes were found to loose considerable amount of both these surface carbohydrate moieties in the epididymis. In fact, in the case of non-amino neutral sugars, the loss amounted to nearly 50%. This does not come as a surprise since the epididymis is considered to be one of the richest sources of glycosidases and glycosyltransferases (Conchie et al., 1959; Jones and Glover, 1973; Chapman and Killian, 1984 and Hall and Killian 1987). Obviously, these enzymes are present for trimming and processing of the sperm surface carbohydrates, and may be predominantly responsible for the transformation of sperm from the immature to the mature form.

Carbohydrate moieties are associated with the oligosaccharide chains of glycoproteins, glycocalyx and glycolipids of the sperm surface. The presence of specific configuration of sugar components has been reported to be essential for the biological activity and survival of certain glycoproteins during epididymal transit (Acott et al., 1979; Dacheux et al., 1983; Usselman et al., 1985; Hamilton et al., 1986; Ryan et al., 1987; Fournier Delpech et al., 1988; Toshimori et al., 1988; Reynolds et al., 1989; Sylvester et al., 1989; Danzo and Black, 1990a,b; Delgado et al., 1990; Topfer - Petersen et al., 1990 and Sensibar et al., 1993) The role of sugar moieties in cell - cell interaction, including those involving sperm and egg (Miller and Ax, 1990), has assumed great importance in recent years. Sugars could act as receptors for species specific recognition of gametes and at times even play a protective role. Thus, there seems every possibility that the changes in the sperm surface sugar components are associated with epididymal sperm maturation and cannot be taken lightly (Macek and Shur, 1988, Boldt et al., 1989; Jones, 1989b and Jonakova et al., 1991).

It is known that sialic acids are synthesized in the epididymis and transferred to the surface of sperm present within its lumen. Holt (1980) reports that the sialic acid present on ejaculated sperm is acquired from the epididymis. The abundance of sialyltransferases in the epididymal duct facilitates this process (Durr et al., 1977; Bernal et al., 1980 and Tulsiani et al., 1993). Simultaneously, desialylation is also believed to occur since high levels of sialidase activity has been detected in this organ. In the present study it has been observed that the level of

sialic acids on sperm membrane continuously falls as sperm is transported from the caput to the cauda epididymis. The sialic acid composition of sperm membranes decreased to as much as 20% in cauda sperm compared to what was present on caput sperm. From these results it appears that of the two mechanisms, sialylation is much higher in the caput while desialylation predominates in the cauda. Bernal et al., (1980) also subscribe to this view. This fact is also supported by a number of previous investigations where sialic acid measurements have been made in epididymal luminal fluid collected from various sites along the epididymis (Rajalakshmi et al., 1968).

Sialic acids are believed to contribute substantially to the negative charge of sperm (Bedford, 1963; Bedford et al., 1973; Moore, 1979 and Holt, 1980). It has been reported that sperm acquire an increasing negative charge as they move from the caput to cauda. This logically means that there should be an increase in the level of sialic acid on the sperm surface. On the contrary, what is observed is a lower level of sialic acid in cauda sperm compared to caput sperm. At this stage, it would be useful to recall that processes involved in both addition and removal of sialic acids occur simultaneously on epididymal sperm (Bernal, 1980 and Holt, 1980). It is likely that the operation of both mechanisms at the same time helps in bringing cryptic sialic acid residues to the sperm surface (Jones, 1989b). Although there is a quantitative loss of sialic acid on the sperm surface, the exposition of cryptic sialic acid residues is perhaps reflected in the increase in negative charge as observed by other investigators. This view is further substantiated by the fact

that WGA, a lectin which binds to N-acetylglucosamine dimers or sialyl residues, bound weakly to ram caput sperm. This binding intensity was found to increase as sperm passed through the epididymis, and persists in ejaculated sperm. Neuraminidase treatment of cauda sperm reduces the level of WGA binding (Yanagimachi et al., 1972 and Magargee et al., 1988).

In the present study the total proteins on sperm plasma membrane were also found to decrease during passage through the epididymis. Many factors could contribute to the loss in protein. Previous studies have pointed out that the protein profiles on sperm do not remain constant and in fact change dynamically during their transit through the epididymis (Bostwick et al., 1980; Jones et al., 1981; Olson and Orgebin - Crist, 1982; Dacheux and Voglmayr, 1983; Voglmayr et al., 1983 and Eddy, 1988).

Some investigators have identified specific proteins that are shed from the surface of sperm (Voglmayr et al., 1980, 1983; Brooks, 1981b; Fraser, 1984 and Jones, 1989b). In the present situation, since the level of carbohydrates is also reduced substantially along with proteins, it is likely that the molecules lost from the sperm surface are atleast in part glycoproteins. Yet another explanation proposed is that the same protein, while remaining anchored on the sperm surface, could be cleaved by the action of proteases (Kennedy and Polakoski, 1981; Blobel et al., 1990 and Phelps et al., 1990). In fact, the presence of proteases has been demonstrated in the epididymis (Dott and Dingle, 1968; Danzo, 1986 and Hendry and Danzo, 1986). There is also a possibility that the reduction in the protein level reflects a loss of membrane itself. Hoffmann and Killian (1981) speculate that this might be the

precise reason for the fact that the protein to phospholipid composition remains the same in sperm from the different segments of the epididymis.

Thus, from the present investigations there is ample evidence to suggest that the plasma membrane of sperm is extensively modified in the epididymis. The quantum of changes could well be the difference between an immature and mature sperm.

3.5 CONCLUDING REMARKS

1. A novel procedure has been described for the simultaneous separation of sperm from luminal fluid and tissue using goat epididymis.

2. Exposure of tissue slices to vacuum generated by an evacuation pressure of 200 mm Hg for 2 min followed by filtration through cheesecloth forms the essence of the procedure.

3. By this technique sperm free of contamination from other epithelial cells could be obtained from virtually any region of the epididymal duct. Sonication of sperm followed by a series of centrifugation steps on sucrose gradients yields large amounts of membrane fractions.

4. Electron microscopic studies (using a negative staining) and adenyl cyclase estimation confirmed the purity of the sperm plasma membrane.

5. Biochemical analysis was done on plasma membrane obtained from sperm collected at twelve sites along the epididymal duct.

6. The influence of the epididymal microenvironment on sperm is seen from major changes in the carbohydrate, lipid and protein composition of sperm membranes while passing through the epididymis. An attempt has been made to analyse the factors that influence the changes in biochemical composition of sperm surface in relation to epididymal sperm maturation.

7. A general depletion in the levels of total protein, sialic acid, neutral sugars, amino sugars, phospholipid and cholesterol in sperm membranes is an indicator of an extensive remodelling of the sperm surface during epididymal transit.

CHAPTER-2
SOLUBILIZATION OF SPERM
MEMBRANE PROTEINS

4.1 INTRODUCTION

It is well known that the basic structure of biological membranes is determined by the lipid bilayer, while their specific functions are determined largely by proteins. Therefore, it is not surprising that in sperm too, membrane proteins have received the attention of many investigators in recent times. The extensive remodelling of sperm surface proteins, reported during epididymal maturation, may be related to functional changes.

Based on the type of association of the protein, with the lipid bilayer environment of the membrane and the procedure employed for their release, the membrane proteins have been put into three categories: (a) loosely bound proteins which may be transiently located in the membrane and are easily removed by changes in salt concentrations, (b) extrensic or periferal proteins which are not released easily from the membranes and can be removed by changing the electrostatic environment of the membrane; (c) tightly bound intrinsic or integral proteins which can be recovered from the membrane only under conditions that disturb the hydrophobic interactions and lead to disruption of membrane. Many proteins extend across the lipid bilayer and are exposed to anaqueous environment on both sides, and are referred to as transmembranal proteins. Other proteins are exposed to the aqueous medium only on one side of the bilayer; some of these are anchored to the membrane by noncovalent interactions, for example transmembrane proteins, while others may be anchored by means of covalently attached fatty acid chains that extend into one monolayer or the other.

For detailed studies involving membrane proteins it is imperative to dislodge these proteins from other membrane components. Several methods are in use for the release of proteins from membranes. In summary the strategies used include:

a) Changes in the electrostatic conditions of the membrane's environment forexample by changes in ionic strength using chelating agents, reducing agents and pH

b) Modifications of ionogenic groups such as sulfhydryl groups or acid labile groups

c) Protein perturbants - chaotropic agents such as urea, guanidine hydrochloride, lithium diiodosalicylate

d) Enzymes such as proteases or galactose oxidase

e) Sonication

f) Organic solvents

g) Detergents, both ionic and nonionic.

Since, the current study is directed towards the protein components of sperm membranes and their modification during epididymal transit, attempts have been made to solubilize sperm membrane proteins using two ionic and two nonionic detergents. These is no emperical guideline relating to the use of detergents, each system having its own unique set of conditions best suited for the nature of membrane or the type of protein under consideration. Therefore, goat sperm membrane proteins have been solubilized using sodium dodecyl sulfate, sodium deoxycholate, Nonidet P-40 and Triton X-100. Conditions have been optimized to see which of these detergents are useful for dealing with sperm membrane proteins.

4.2 MATERIALS AND METHODS

Four detergents sodium dodecyl sulfate (Boehringer Mannheim), sodium deoxycholate (Loba), Nonidet P-40 (Sigma) and Triton X-100 (Boehringer Mannheim) are used for solubilization studies. Stock solutions of these detergents are made in suitable buffers as specified below:

Stock solution of 10% sodium dodecyl sulfate: 5g sodium dodecyl sulfate is dissolved in 50ml 0.0625M Tris-HCl buffer (pH6.8) containing 1mM PMSF.

Stock solution of 10% sodium deoxycholate: 5g sodium deoxycholate is dissolved in 50ml 0.02M Tris buffered saline (pH8.0) containing 1mM PMSF.

Stock solution of 10% Triton X-100: 5ml Triton X-100 in 50ml final volume of Tris-HCl (pH 6.8 - 7.0) containing 1mM PMSF.

Stock solution of 10% Nonidet P-40: 5 ml Nonidet P-40 in 50ml final volume of 10mM HEPES (pH7.4), 0.15M NaCl containing 1mM PMSF.

4.2.1 Optimization of Solubilization Condition

The optimal concentration of detergent, time of incubation and the detergent to protein ratio have been worked out for each of the detergents using the protocols described below.

4.2.1.1 Solubilization with Sodium dodecyl sulfate

Sperm membrane preparation containing a total of 0.5 mg protein are used for this set of studies. Solubilization of membrane

protein is attempted using 0.1, 0.2, 1.0, 2.0 and 5.0% sodium dodecyl sulfate in 0.0625M Tris-HCl (pH6.8). It has been observed in general practice that the efficiency of protein extraction with sodium dodecyl sulfate varies with temperature. The time required for extraction is considerably reduced with an increase in temperature. Thus, membrane protein is extracted under two sets of conditions. At $60^{\circ}C$, prolonged periods of incubation are used (15 min, 30 min, 1 hour and 2 hours); while at $100^{\circ}C$, incubation time is shorter (1 min, 3 min, 10 min and 30 min). At the end of incubation the detergent mixture is centrifuged (10,000 xg for 10min) and the solubilized proteins recovered in the supernatant.

The efficiency of protein extraction using sodium dodecyl sulfate is also investigated in sperm membrane preparations containing a total of 0.5mg, 1mg, 2mg and 3mg protein. For this purpose solubilization is carried out at 0.1, 0.2, 1.0, 2.0 and 5.0% sodium dodecyl sulfate concentration in 0.0625M Tris-HCl (pH 6.8) at $100^{\circ}C$ for 3min. The solubilized proteins are recovered in the supernatant after centrifugation (10,000 xg for 10min).

4.2.1.2 Solubilization with Sodium deoxycholate

Varying concentrations of sodium deoxycholate (0.1, 0.2, 1.0, 2.0 and 5.0%) in 0.02M Tris buffered saline (pH 8.0) containing 1mM PMSF are used to solubilize proteins from membrane fractions containing a total a 0.5mg protein. Extractions are carried out at room temperature for 15 min, 30 min, 1 hour and 2 hour. The solubilized proteins are recovered after centrifugation (10,000 xg for 10min).

Solubilization using 0.1, 0.2, 1.0, 2.0 and 5.0% sodium deoxycholate is performed in membrane preparations containing higher protein concentrations (0.5mg, 1mg, 2mg and 3mg) at room temperature for 30 min. The solubilized proteins are recovered after centrifugation (10,000 xg for 10min).

4.2.1.3 Solubilization with Triton X-100

Several concentrations of Triton X-100 (0.1, 0.25, 0.5, 1.0 and 5.0%) in 0.02M Tris-HCl (pH 6.8) containing 1mM PMSF are used to solubilize sperm proteins from a membrane preparations containing a total of 0.5mg protein. The incubation is carried out on ice for 15 min, 30 min, 1 hour and 2 hour. Just as in previous cases, solubilization is attempted at higher membrane protein concentrations (0.5mg, 1mg, 2mg and 3mg) on ice for 1 hour. The solubilized proteins are recovered after centrifugation (10,000 xg for 10min).

4.2.1.4 Solubilization with Nonidet P-40

Nonidet P-40 at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0% in 10mM HEPES buffer (pH 7.4) containing 0.15M NaCl and 1mM PMSF is used to solubilize sperm membrane proteins from membrane preparations containing a total of 0.5mg protein. Incubation is carried out at room temperature for 15 min, 30 min, 1 hour and 2 hours. Using the same range of detergent concentrations, the efficiency of extraction is monitored in membrane preparations containing 0.5mg, 1mg, 2mg and 3mg protein for 30 min at room temperature. The solubilized proteins are recovered in the supernatant by centrifugation (10,000 xg for 10min).

4.2.2   Estimation of the Protein Concentration in Fractions Solubilized from Membranes

The conventional method of Lowry et al., (1951) for the estimation of detergent solubilized proteins was found to be inappropriate, owing to the interference of detergents with the estimation method.   Alternative methods have therefore been used.   The quantitation of proteins present in samples solubilized with sodium dodecyl sulfate, sodium deoxycholate and Triton X-100 was determined by the method of Peterson (1977).   On the other hand, estimation of solubilized proteins in the case of Nonidet P-40 was done employing the Bicinchoninic Acid (BCA) method of Smith et al., (1985).

4.2.2.1 Estimation of protein by Peterson (1977) method

This method is a modification of the original method of Lowry et al.,(1951).   It is rapid, simple, objective and generally more applicable to solubilized proteins from membrane.   The precipitation of proteins using sodium deoxycholate – trichloroacetic acid is used for rapid and quantitative recovery of soluble and membrane proteins from interfering substances, even in very dilute solutions (< 1 µg/ml of protein). Sodium dodecyl sulfate is added to alleviate possible nonionic and cationic detergent and lipid interferences, and to provide mild conditions for rapid denaturation of membrane and proteolytic proteins.

Reagents

1.   Copper-Tartrate-Carbonate (CTC) solution: A solution of 0.1% copper sulfate pentahydrate, 0.2% potassium tartrate and 10% sodium carbonate.

(a) Copper-Tartrate solution: 0.08g copper sulfate pentahydrate and 0.16g potassium tartrate dissolved in 40ml water.

(b) 10% Sodium carbonate: 8.0g sodium carbonate dissolved in 40ml water.

The sodium carbonate is added slowly with mixing to the copper tartrate solution.

2. 5% Sodium dodecyl sulfate: 8.0g sodium dodecyl sulfate dissolved in a final volume of 160ml water.

3. 0.8M Sodium hydroxide : 2.56g sodium hydroxide in 80ml water.

Reagent A: This is prepared by mixing one part of the copper-tartrate-carbonate solution, two parts of sodium dodecylsulfate solution and one part of sodium hydroxide solution. This reagent is stable for 2-3 weeks and the white precipitate formed due to the presence of SDS at cooler temperatures is easily dissolved on warming. The accumulation of dark precipitate however is not suitable and the reagent is not used as it affects color development.

Reagent B: One part 2N Folin-Ciocalteau Phenol reagent is mixed with five parts distilled water. This reagent is stable for a month in a dark bottle.

Solutions for protein precipitation

1. 0.15% Sodium deoxycholate: 0.075g sodium deoxycholate dissolved in 50ml distilled water.

2. 72% Trichloroacetic acid: 7.2g trichloracetic acid in 10ml distilled water.

3. Bovine serum albumin (0.5mg/ml): Fraction V bovine serum albumin (Sigma) is used and 10mg bovine serum albumin is dissolved in 20ml water.

Procedure

(a) To samples containing between 5 to 100μg protein in a total volume of 1.0ml made up in distilled water, 0.1ml of 0.15% sodium deoxycholate is added, mixed and allowed to stand for 10 min at room temperature. 0.1ml of 72% trichloroacetic acid is added next, mixed and centrifuged at 3000xg for 15 min. The supernatant is discarded by decanting and upending the tube on a paper towel. The supernatant adhering to the sides of the tube is removed by aspiration.

(b) The pellet obtained in the above step is dissolved in 1ml water. To this 1.0ml of Reagent A is added and mixed. After 10min at room temperature 0.5ml Reagent B is added and mixed rapidly. The samples so treated are incubated at room temperature for 30 min. The color developed is read against a reagent blank at 750nm on a Beckman DU-6 spectrophotometer. The standard plot is worked out using the values obtained for bovine serum albumin at concentrations between 10 to 100 μg

4.2.2.2 Bicinchoninic acid assay of proteins (Smith et al., 1985)
Bicinchoninic acid sodium salt is a stable, water soluble compound capable of forming an intense purple complex with cuprous ion (Cu^+) in an alkaline environment. This reagent forms the basis of an analytical method capable of monitoring cuprous ion produced in the reaction of protein with alkaline Cu^{2+} (biuret reaction). The

color produced from this reaction is stable and increases in a proportional fashion over a broad range of increasing protein concentrations. It is an assay method more tolerable to detergents and ions in ranges over and above those by Lowry's method. bicinchoninic Acid assay kit (Pierce) is used.

Reagents

Reagent A: An aqueous solution of 1% bicinchoninic acid, 2% sodium carbonate. H_2O, 0.16% sodium tartrate, 0.4% sodium hydroxide and 0.95% sodium bicarbonate. The pH of the solution is adjusted to 11.25, if need be by sodium hydroxide (50%) or sodium bicarbonate (solid).

Reagent B: 4% Copper sulfate pentahydrate in deionized water.

The Standard working reagent: Prepared by mixing 100 volumes of Reagent A with 2 volumes of Reagent B. The reagent is apple green in color.

Procedure

To samples in 0.1ml final volume, 2.0ml of the standard working reagent is added. The solutions are mixed and absorbance recorded after a 30 minutes incubation at $37^{\circ}C$, at 562nm on a Beckman DU-6 spectrophotometer. The optical density obtained for standard BSA (10 to 100µg) concentration at 562nm against a reagent blank is used for the calibration curve.

4.2.3 Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The sperm membrane proteins solubilized using these different detergents are qualitatively analysed on SDS-PAGE (Laemmli, 1970).

Reagents

Acrylamide : Bisacrylamide (30:0.8): 30.0g acrylamide and 0.8g NN' methylene bisacrylamide dissolved in distilled water and final volume made to 100ml. The solution is filtered through whatman filter paper No.1 and stored in a dark bottle at 4°C.

Resolving gel buffer (3.0M Tris-HCl, pH 8.8) : 36.3g trishydroxymethane aminomethane dissolved in 48ml of 1M HCl and pH adjusted to 8.8 using 1 M HCl. The volume is made upto 100ml with water and stored at 4°C.

Stacking gel buffer (0.5M Tris-HCl, pH 6.8) : 6.0g Tris hydroxymethane aminomethane dissolved in 40ml distilled water and titrated to pH 6.8 with 1M HCl. The solution is filtered through whatman filter paper No.1 and stored at 4°C.

Ammonium persulfate (1.5% w/v) : 0.15g Ammonium persulfate dissolved in 10ml distilled water. This solution is always prepared fresh before use.

10% (w/v) Sodium dodecyl sulfate: 10g sodium dodecyl sulfate dissolved in a final volume of 100ml distilled water.

TEMED: N,N, N',N', tetramethyleneethylenediamine is used as supplied.

Staining the gels: Gels are stained overnight in 0.1% Coomassie brilliant blue in 40% methanol and 10% acetic acid.

Destaining: The staining solution is drained and the gels are rinsed in distilled water briefly. The gels are then destained using 10% acetic acid and 5% methanol.

Casting the gel

A 14x16x0.15cm, 10% SDS-PAGE is cast as given below:

	Stacking gel (in ml)	Resolving gel (in ml)
Acrylamide: Bisacrylamide (30:0.8)	2.5	10.0
Stacking gel buffer	5.0	–
Resolving gel buffer	–	3.75
1.5% (w/v) Ammonium per sulfate	1.0	1.5
10% (w/v) sodium dodecyl sulfate	0.2	0.3
Water	11.3	14.45
TEMED	0.015	0.015

4.3 RESULTS

Attempts at solubilizing protein from sperm membrane with sodium dodecyl sulfate gives valuable information on the nature of the interaction of this detergent with sperm membrane. It is clear from the data (Fig. 4.1) that the solubilization of membrane protein is enhanced steadily with the increase in SDS concentration to 2.0% at $100^{o}C$ beyond this there is not much improvement in the solubilizing capacity of this detergent. At $60^{o}C$ it appears that exposure of sperm membrane to 1.0% SDS for 1h is most effective in solubilizing proteins (Fig. 4.2). Around 48% of the membrane protein is solublized. However, the solubilizing ability of the same detergent is significantly enhanced when the temperature is raised to $100^{o}C$. The time required for solubilization is also considerably less. At this temperature 2% SDS is able to solubilize as much as 98% of the sperm membrane proteins in just three minutes.

Sodium deoxycholate solubilized of sperm membrane proteins with limited success (Fig. 4.3). Exposure of membrane preparations to this detergent improved the solubilization with time over the first 30 min. Extending the period of incubation does not enhance solubilization . Of the several concentrations of detergent experimented with, 1% sodium deoxycholate produces the best results after a 30min incubation time, solubilizing 65% of the membrane proteins.

The efficiency of Nonidet P-40 in solubilizing sperm membrane proteins, when compared to the ionic detergents, is quite limited (Fig.4.4). Even under the most optimal conditions, this detergent could at best solubilize less than 30% of the sperm membrane proteins.Any deviation of solubilization conditions from the optimal state of 1% detergent concentration and incubation time of 30 min reduces the quantity of proteins solubilized. Enhancement of detergent concentration and the time of exposure did not affect the quantity of proteins being solubilized.

The solubilization efficiency of Triton X-100 is similar to that of Nonidet P-40, with Triton X-100 exhibiting a marginal increase in the quantity of proteins being solubilized (Fig.4.5). The optimal solubilization conditions for Triton X-100 were at a detergent concentration of 0.5% and incubation time of 1h on ice. There is an increase in protein solubilization with an initial increase in detergent concentration and incubation. However, extending incubation time beyond 1h and increasing detergent concentration to greater than 0.5% does not improve solubilization.

The data on the effectiveness of different detergents in solubilizing proteins from membranes with differences in protein composition are consolidated in Figs. 4.6 to 4.9. It is apparent that SDS and sodium deoxycholate are better than Nonidet P-40 and Triton X-100. Under ideal conditions both SDS and sodium deoxycholate are able to solubilize substantially greater amounts of protein. However, the percentage of proteins solubilized declines with the increase in initial protein concentration beyond a certain point.

From the data presented so far, it is abundantly clear that sodium dodecyl sulfate performs best at a concentration of 2% at $100^{o}C$ for 3 min. Treatment of sperm membrane preparations with 1% Sodium deoxycholate for 30 min at room temperature is found to be ideal. Maximum protein solubilization using Nonidet P-40 is obtained at a concentration of 0.5% after 30 min exposure to the detergent at room temperature. The use of 0.5% Triton X-100 at $0^{o}C$ for 1h on ice is the optimal set of conditions to achieve maximal solubilization with this detergent.

When sperm membrane proteins are solubilized by detergents under these sets of conditions and qualitatively analysed on 10% SDS-PAGE certain interesting features come to light.

The electrophoretogram of proteins solubilized by 2% SDS at $100^{o}C$ for 3 min from the membrane preparations of the sperm obtained from the twelve segments of the epididymis is given in Fig. 4.10. From the electrophoretogram it is apparent that there is an increase in the staining intensity of the low molecular weight

Fig. 4.1   Effect of Varying Incubation Time on Solubilization of
            Sperm Membrane Proteins Using Sodium Dodecyl Sulfate at
            100°C.   The amount of protein solubilized from 0.5mg
            sperm membrane preparations after exposure to sodium
            dodecyl sulfate for (A) 1 min, (B) 3 min, (C) 10 min and
            (D) 30 min is expressed as µg.   The bars represent the
            data obtained when (▬▬) 0.1%, (▭) 0.2%, (▱)
            1.0%, (▤) 2.0% and (▥) 5.0% sodium dodecyl
            sulfate is used for solubilization.

Fig. 4.2   Effect of Varying Incubation Time on Solubilization of
            Sperm Membrane Proteins Using Sodium Dodecyl Sulfate at
            60°C.   The   amount of protein solubilized from 0.5mg
            sperm membrane preparations after exposure to sodium
            dodecyl sulfate for (A) 15 min, (B) 30 min, (C) 1 h and
            (D) 2 h is expressed as µg.   The bars represent the data
            obtained when (▬▬) 0.1%, (▭) 0.2%, (▱) 1.0%,
            (▤) 2.0% and (▥) 5.0% sodium dodecyl sulfate is
            used for solubilization.

Fig. 4.3   Effect of Varying Incubation Time on Solubilization of
            Sperm Membrane Proteins Using Sodium Deoxycholate at
            Room Temperature.   The   amount of protein solubilized
            from 0.5mg sperm membrane preparations after exposure to
            sodium deoxycholate for (A) 15 min, (B) 30 min, (C)
            1 h and (D) 2 h is expressed as µg.   The bars represent
            the data obtained when (▬▬) 0.1%, (▭) 0.2%,
            (▱) 1.0%, (▤) 2.0% and (▥) 5.0% sodium dodecyl
            sulfate is used for solubilization.

FIG. 4·1

FIG. 4·2

FIG. 4·3
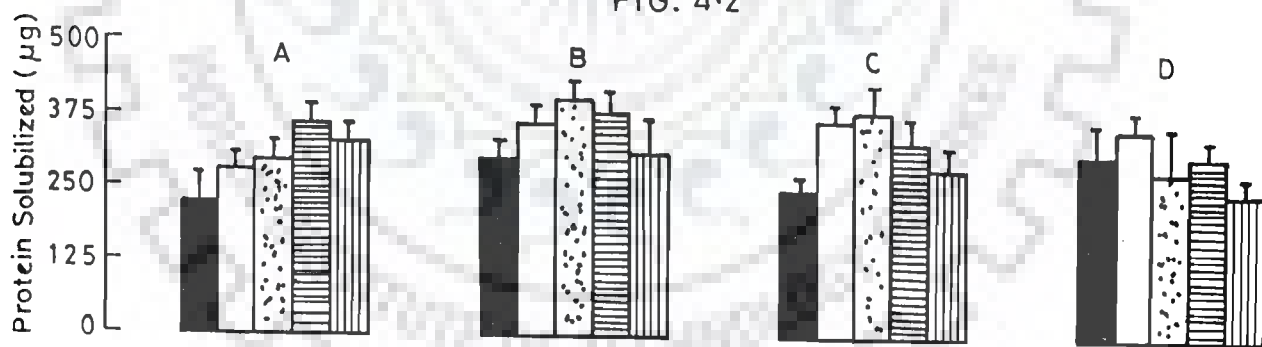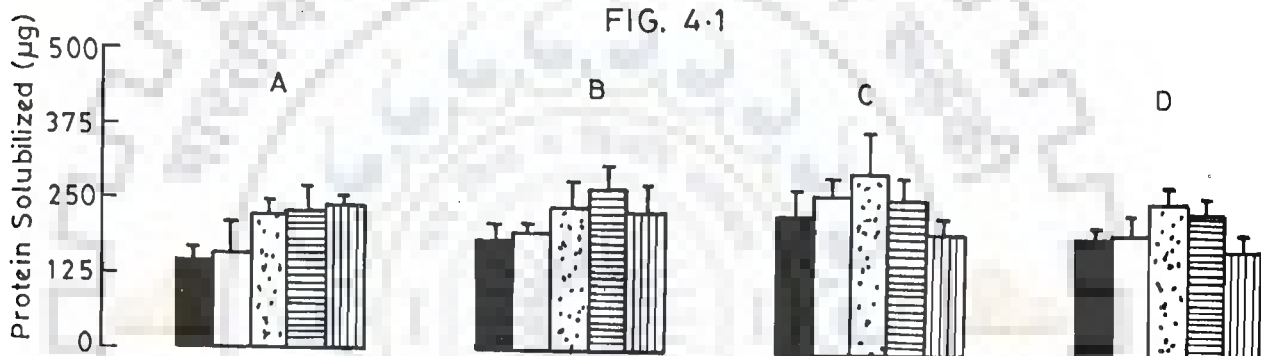
Fig. 4.4   Effect of Varying Incubation Time on Solubilization of Sperm Membrane Proteins Using Nonidet P-40 at Room Temperature. The amount of protein solubilized from 0.5mg sperm membrane preparations after exposure to Nonidet P-40 for (A) 15 min, (B) 30 min, (C) 1 h and (D) 2 h is expressed as μg. The bars represent the data obtained when (▬) 0.1%, (▭) 0.2%, (▦) 0.5%, (▤) 1.0% (▥) 2.0% and (▨) 5.0% Nonidet P-40 is used for solubilization.

Fig. 4.5   Effect of Varying Incubation Time on Solubilization of Sperm Membrane Proteins Using Triton X-100 at 4°C. The amount of protein solubilized from 0.5mg sperm membrane preparations after exposure to Triton X-100 for (A) 15 min, (B) 30 min, (C) 1 h and (D) 2 h is expressed as μg. The bars represent the data obtained when (▬) 0.1%, (▭) 0.25%, (▦) 0.5%, (▤) 1.0% and (▥) 5.0% Triton X-100 is used for solubilization.
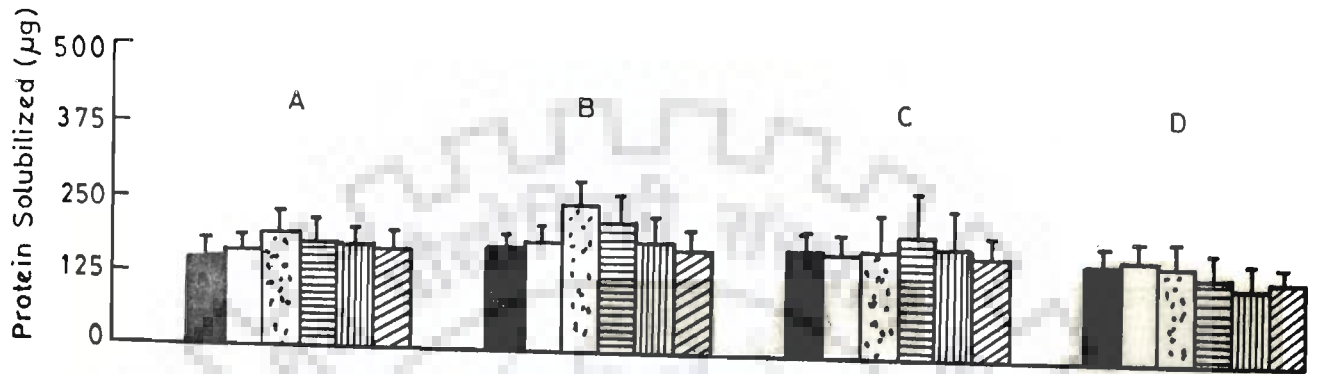
FIG. 4.4
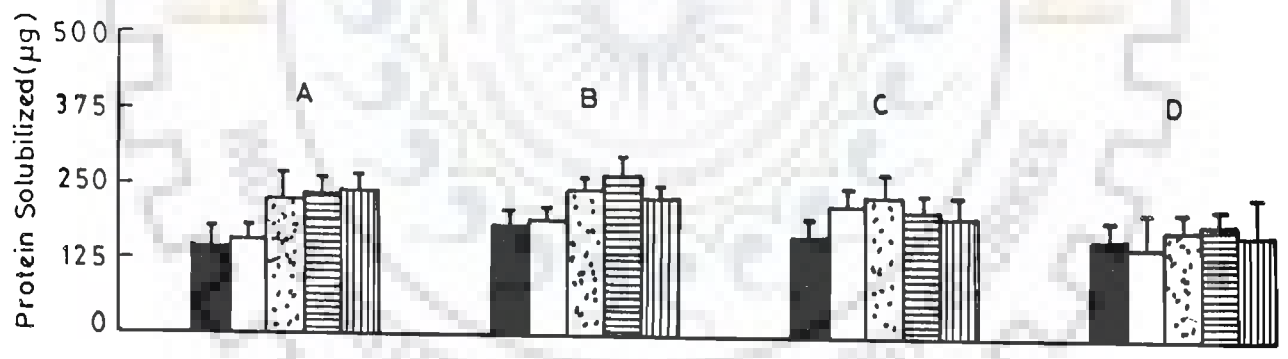
FIG. 4.5

Fig. 4.6   Effect of Varying Initial Protein Concentration on
            Solubilization of Sperm Membrane Proteins Using Sodium
            Dodecyl Sulfate at $100^{\circ}C$ for 3 min. The amount of
            proteins solubilized from sperm membrane preparations
            containing (A) 0.5 mg (B) 1.0 mg (C) 2.0 mg and (D) 3.0
            mg is expressed as μg. The bars represent the data
            obtained when (■■■) 0.1%, (▭) 0.2%, (▨)
            1.0%, (☰) 2.0% and (⊞⊞⊞) 5.0% Sodium dodecyl
   sulfate is used for solubilization.

Fig. 4.7   Effect of Varying Initial Protein Concentration on
            Solubilization of Sperm Membrane Proteins Using Sodium
            Deoxycholate at room temperature for 30 min. The amount
            of proteins solubilized from sperm membrane preparations
            containing (A) 0.5 mg (B) 1.0 mg (C) 2.0 mg and (D) 3.0
            mg is expressed as μg. The bars represent the data
            obtained when (■■■) 0.1%, (▭) 0.2%, (▨)
            1.0%, (☰) 2.0% and (⊞⊞⊞) 5.0% Sodium dodecyl
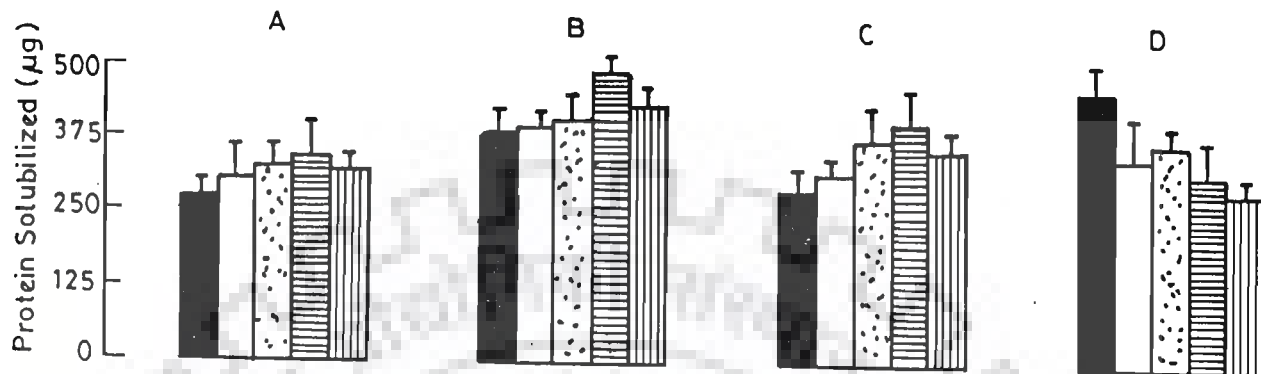   sulfate is used for solubilization.
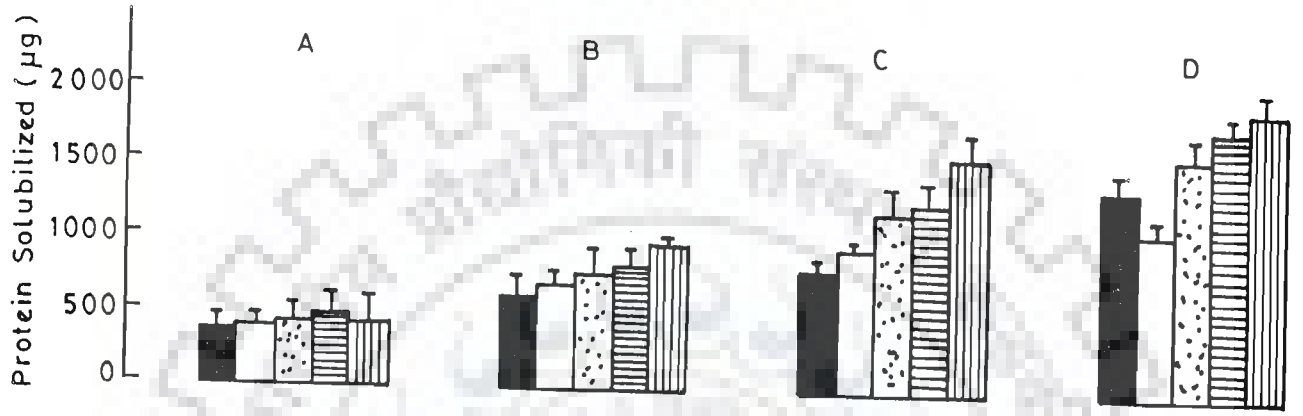
FIG. 4.6
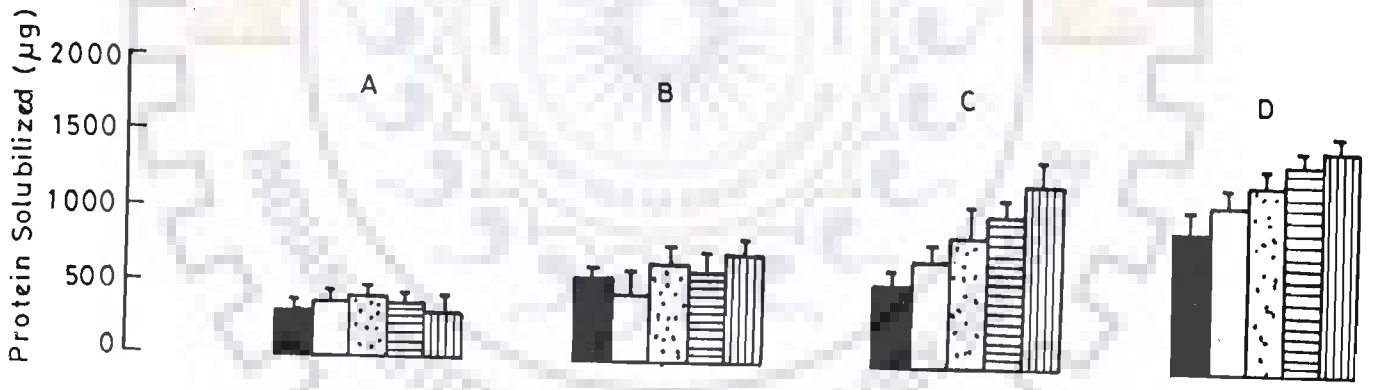
FIG. 4.7

Fig. 4.8   Effect of Varying Initial Protein Concentration on
            Solubilization of Sperm Membrane Proteins Using Triton
            X-100 on Ice for 1 h.   The amount of proteins
            solubilized from sperm membrane preparations containing
            (A) 0.5 mg (B) 1.0 mg (C) 2.0 mg and (D) 3.0 mg is
            expressed as μg.   The bars represent the data obtained
            when (▬) 0.1%, (▭) 0.2%, (▱) 1.0%,
            (▤) 2.0% and (▥) 5.0% Triton X-100 is used for
            solubilization.

Fig. 4.9   Effect of Varying Initial Protein Concentration on
            Solubilization of Sperm Membrane Proteins Using Nonidet
            P-40 at Room Temperature for 30 min.   The amount of
            proteins solubilized from sperm membrane preparations
            containing (A) 0.5 mg (B) 1.0 mg (C) 2.0 mg and (D) 3.0
            mg protein is expressed as μg.   The bars represent the
            data obtained when (▬) 0.1%, (▭) 0.2%,
            (▱) 0.5%, (▨) 1.0% (▤) 2.0% and (▥)
            5.0% Nonidet P-40 is used for for solubilization.
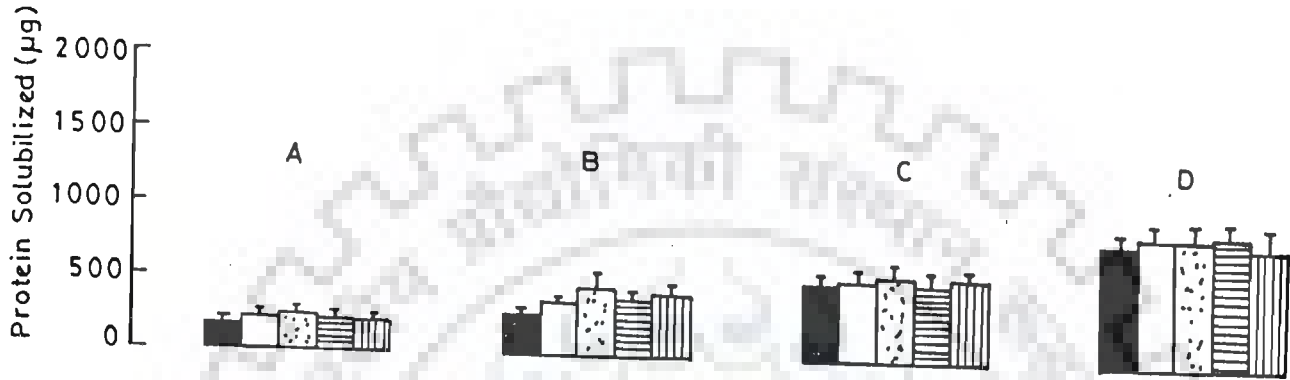
FIG. 4·8
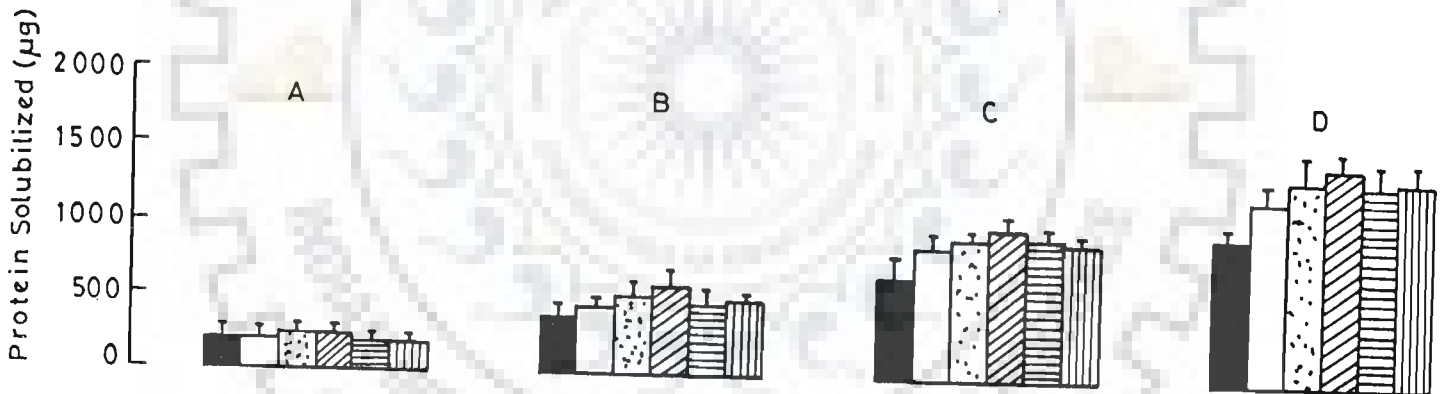
FIG. 4·9

Fig. 4.10   Electrophoretograms   of   Sodium   Dodecyl   Sulfate
Solubilized Sperm Membrane Proteins.   The   protein
profile of the sperm membrane extracted in 2.0% SDS at
$100^{o}C$ for 3 min from the twelve segments of the
epididymis (Lanes 1-12) is presented. 50μg solubilized
protein/well   has   been   electrophoresed   on   10%
discontinuous denaturing SDS-PAGE at 30 mA constant
current and stained with Coomassie brilliant blue.

Fig. 4.11   **Electrophoretograms of Sodium Deoxycholate Solubilized
Sperm Membrane Proteins.**   The protein profile of the
sperm membrane extracted in 1.0% Sodium deoxycholate at
room temperature for 30 min from the twelve segments of
the   epididymis   (Lanes   1-12)   is   presented.   50μg
solubilized protein/well has been electrophoresed on 10%
discontinuous denaturing SDS-PAGE at 30 mA constant
current and stained with Coomassie brilliant blue.

kD
97·4 →
66 →
45 →
29 →
14·4 →

LANE No.　1　2　3　4　5　6　7　8　9　10 11 12

FIG. 4·10

kD
97·4 →
66 →
45 →
29 →
14·4 →

LANE No.　1　2　3　4　5　6　7　8　9　10　11　12
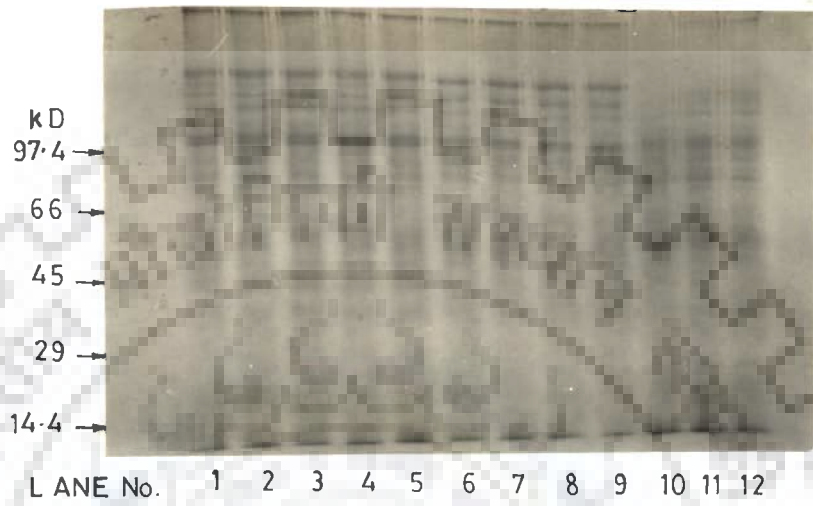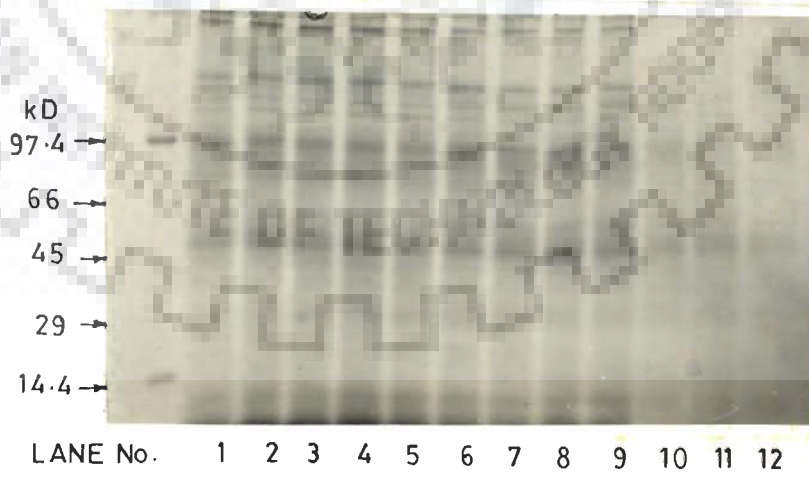
FIG. 4·11

Fig. 4.12   **Electrophoretograms of Triton X-100 Solubilized Sperm Membrane Proteins.**   The protein profile of the sperm membrane extracted in 0.5% Triton X-100 on ice for 1h from the twelve segments of the epididymis (Lanes 1-12) is presented. 50µg solubilized protein/well has been electrophoresed on 10% SDS-PAGE at 30 mA constant current and stained with Coomassie brilliant blue

Fig. 4.13   **Electrophoretograms of Nonidet P-40 Solubilized Sperm Membrane Proteins.**   The protein profile of the sperm membrane extracted in 0.5% Nonidet P-40 at room temperature for 30 min from the twelve segments of the epididymis (Lanes 1-12) is presented.   50µg solubilized protein/well has been electrophoresed on 10% denaturing, discontinuous SDS-PAGE at 30 mA constant current and stained with Coomassie brilliant blue.

kD
97·4 →
66 →
45 →
29 →
14·4 →

LANE No.   1   2   3   4   5   6   7   8   9   10   11   12

FIG. 4·12

kD
97·4 →
66 →
45 →
29 →
14·4 →
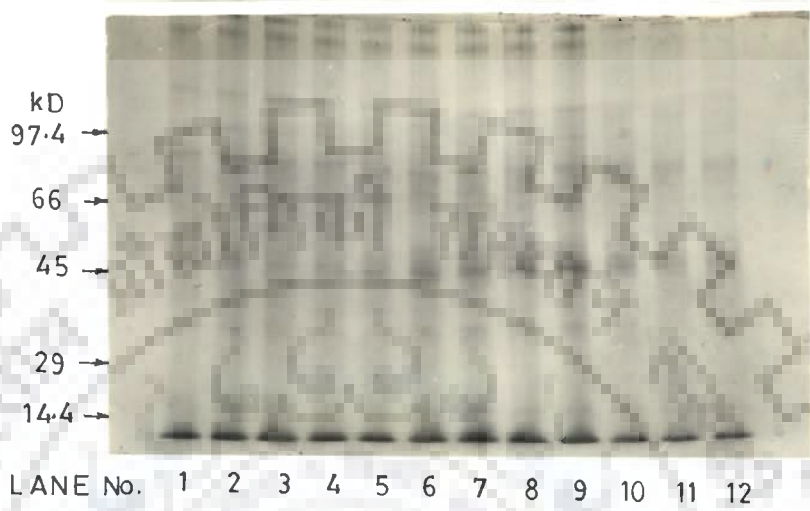
LANE No.   1   2   3   4   5   6   7   8   9   10   11   12
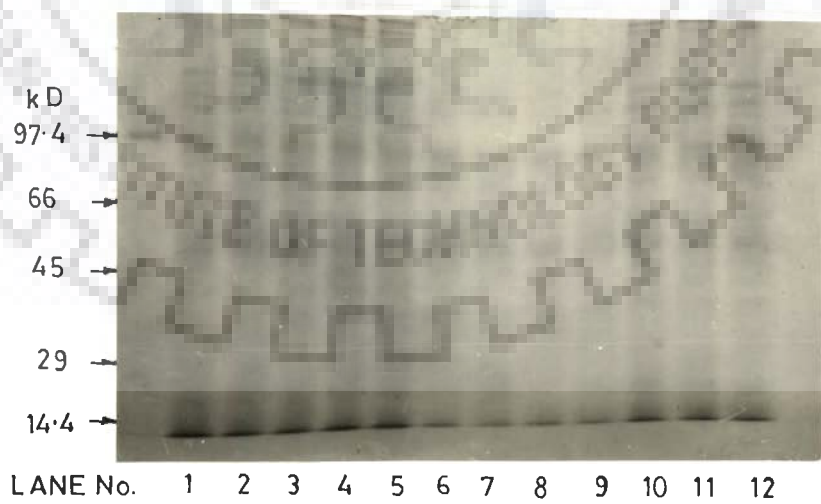
FIG. 4·13

components in the distal segments (10, 11 and 12) while the population of high molecular weight proteins is prominent in the initial segments. The proteins solubilized by 1.0% sodium deoxycholate for 30 min at room temperature are given in Fig. 4.11. The pattern of the protein profile as similar to sodium dodecyl sulfate. However, the difference lies in the absence of certain intensely staining bands of Mr < 45kD present in the protein profiles of SDS solubilized sperm membrane fractions from the twelve segments of the epididymis. The protein profiles of Triton X-100 and Nonidet P-40 are given in Figs. 4.12 and 4.13. The solubilization by Nonidet P-40 gives a very sparse population of proteins while the Triton X-100 solubilized proteins are more abundant. However, there is a distinct reduction in the number of protein bands resolved in case of the two nonionic detergents.

4.4 DISCUSSION

Most tightly bound membrane proteins can be solubilized by agents that disrupt hydrophobic association and destroy the bilayered structure of the membrane (Heleneius and Simons, 1975; Tanford, 1980 and Neugebauer, 1990). One such agent is detergent, which by virtue of being a small amphipathic molecule, tends to form micelles in water. The solubilization obtained with both ionic and non ionic detergents amply demonstrates that these agents could be powerful tools that help in solubilizing proteins from their membrane anchors.

Scientists have often been successful in isolating and solubilizing specific proteins of interest, at times in their

native biologically active state, by judicious choice of detergents. SDS, sodium deoxycholate, Nonidet P-40 and Triton X-100 are the ones that have found extensive application in this regard. The use of SDS for solubilizing sperm surface proteins has been extensive (Ji et al., 1981; Brandt and Hoskins, 1980; Voglmayr et al., 1985; Hamilton et al., 1986; Peterson et al., 1989; Shalgi et al., 1989 and Bellve et al., 1992). Nonidet P-40 has been used to solubilize mouse sperm components (Toshimori et al., 1990b). Sodium deoxycholate has been used to solubilize a post vasectomy autoimmunogen from rat sperm (Handley et al., 1988). Triton X-100 has been used to solubilize a set of PAS positive components from mouse sperm (Rankin et al., 1989). Thus, detergents at appropriate concentrations, to obtain selective proteins for investigation from membranes, are important chemicals for membrane protein studies.

Since the purpose of the present investigation is to extract maximal amounts of sperm membrane proteins, solubilization has been attempted using two ionic (sodium dodecyl sulfate and sodium deoxycholate) and two non ionic (Nonidet P-40 and Triton X-100) detergents. The solubilization efficiency of these detergents is known to be vastly affected by their critical micelle concentration, their aggregation number, the critical micelle temperature, the cloud point and their hydrophile - lipophile balance numbers (Helenius and Simons, 1975). The best conditions for each detergent are established by varying the factors that govern detergent solubilization kinetics. At the outset, the results suggest that the ionic detergents are a better choice for

studying the vast variety of proteins present on the sperm surface. Evidence for such a claim stems from the substantially higher amounts of proteins solubilized from sperm membrane preparations.

Another important observation is that the ionic detergents performed better at higher detergent concentrations relative to the nonionic detergents. This is probably because both the ionic detergents have high cmc values. The cmc of sodium dodecyl sulfate is 8.2mM while that of sodium deoxycholate is 4 to 6mM (Ekwall et al., 1957 and Mukerjee and Mysels, 1971). These critical micelle concentration values indicate that good micelle formation occurs when detergents are used at high concentrations. In contrast, the cmc values of the two non ionic detergents are much lower. For Nonidet P-40 the cmc value is 0.30mM and Triton X-100 has a cmc value of 0.24mM, implying that the micelles are formed at low concentrations (Kushner and Hubbard, 1954). It must be mentioned here that the formation of micelles is not conducive for the solubilization process (Helenius and Simons, 1975). Thus, most workers choose to use detergents below the cmc values.

With the increase in SDS and sodium deoxycholate concentration, significantly greater amounts of membrane protein are found to be solubilized while this was not observed in the case of Nonidet P-40 and Triton X-100. The low cmc values of Nonidet P-40 and Triton X-100 may help explain these results. It appears that once the detergent concentration reaches its cmc value the formation of micelles reduces the availability of detergent molecules to

interact with membranes and remove proteins (Helenius and Simons, 1975 and Neugebauer, 1990).

The effect of temperature on solubilization needs special mention. An interesting inter-relationship between the temperature and time required for solubilization has emerged from studies employing SDS. At $60^{o}C$, the best solubilization is apparent at an incubation time of 1h. However, greater solubilization is achieved at $100^{o}C$ in just 3 minutes. These results demonstrate that for SDS an increase in temperature aids in reducing the time required for protein solubilization from sperm membranes. However the same cannot be said of other detergents, such as Nonidet P-40; sodium deoxycholate and Triton X-100 are more efficient at lower temperatures. Temperature effects on detergent properties is more pronounced in monionic detergents where increasing temperature leads to an exponential expansioni of mixelle (Neugebauer, 1990). Hence, these detergents are used at low temperatures.

From the present study it appears that detergent to protein ratios too, need to be delicately balanced for effective solubilization of membrane proteins. One important finding is that with the increase of initial protein content of membranes, the percentage of solubilization of membrane protein goes down irrespective of the detergent used. This is probably because at higher initial membrane protein concentrations the detergent to protein ratio is low, implying that fewer detergent molecules are available for protein solubilization.

From the electrophoretograms of the proteins solubilized by the four detergents it is apparent that there is a difference in the protein profiles both qualitatively and quantitatively. The best solubilization is achieved by using 2% SDS for 3 min at $100^{\circ}C$ followed by solubilization using sodium deoxycholate. Both the detergents are ionic and the degree of disruption of the bilayered structure of the membrane is more pronounced. This accounts for the dense distribution of protein bands on the electrophoretogram. In contrast, the protein profiles of Nonidet P-40 and Triton X-100 are characterized by a sparse population of bands on the electrophoretograms.

It is abundantly clear from the parameters analysed that solubilization by SDS is superior to other detergents as far as proteins of sperm membrane are concerned. Under optimal conditions, the quantitative amount of proteins solubilized by SDS is higher than that obtained for any other detergent. Moreover, the amount of membrane protein solubilized improved steadily with increasing amount of detergent concentration used. Overall, SDS is able to perform quite well in a wide range of initial protein concentrations of sperm membranes. Even the time required for solubilization of proteins by SDS is low. Electrophoretic analysis of proteins solubilized by SDS gave good resolution. Thus for all practical purposes, SDS is the detergent of choice for solubilizing proteins from sperm membranes.

4.5 CONCLUDING REMARKS

1. Attempts have been made to solubilize sperm membrane proteins using both ionic and non-ionic detergents.

2. The best conditions under which sperm membrane protein could be solubilized have been established.

3. The performance of SDS was ideal at 2% concentration and incubation time of 3 min at 100°C.

4. Incubation of sperm membranes with Sodium deoxycholate for 30 min at room temperature was most effective for protein solubilization.

5. The optimal solubilization conditions for Nonidet P-40 was worked out to be a 30 min exposure to 0.5% detergent concentration at room temperature.

6. A 60 min treatment of sperm membranes with 0.5% Triton X-100 at 4°C was most useful for protein solubilization.

7. A SDS-PAGE separation of proteins solubilized by detergents under these set of optimal conditions produced slab gels with unique staining patterns.

8. Based on the operational convenience and efficiency, solubilization of sperm membrane proteins by SDS is found to be far superior to the other detergents tried.

CHAPTER-3
DESIGNING AND FABRICATION OF ON-LINE ELECTROELUTION APPARATUS

5.1 INTRODUCTION

Offentimes, situations are encountered where a protein found in extremely low concentration must has to be isolated and purified from a complex mixture of other proteins. This is often a tedious process, involving techniques such as ion exchange, gel exclusion, affinity chromatography, isoelectric focussing, isotachophoresis, electrophoresis, and etc. In order to obtain definite results these techniques would have to be judiciously used in the right sequence, depending upon the properties of the protein being purified. A problem, however, arises when detailed properties of proteins being purified are not known.

Many investigators dealing with proteins use electrophoresis in their preliminary analysis and are able to identify the proteins of interest in gels by simple staining techniques. Modification to the original technique such as like using gradient gels and incorporating chemicals like sodium dodecyl sulfate, Cleland's reagents and ampholytes could provide valuable information on the nature of proteins. Thus, polyacrylamide gel electrophoresis has been used as an effective technique for the analysis of proteins (Osterman, 1984; Andrews, 1986 and Hames and Rickwood, 1990). Good resolution is obtained since the proteins, which are separated on the basis of charge, are subjected to molecular sieving by the gel matrix. Despite the excellant resolution obtained, initial efforts to recover the protein of interest from the gel has met with varying degrees of success. But now specialized equipment for electroelution have been developed,

marketed and are currently being used in several laboratories. Although the recovery of proteins from gel has been greatly improved, these equipment are designed for eluting single protein entrapped in gel.

While attempting to study the biochemical basis of sperm maturation in the epididymis, quantitative changes in sperm plasma membrane proteins were observed. Qualitative analysis of sperm plasma membrane by sodium dodecyl sulfate-Polyacrylamide gel electrophoresis revealed distinct protein profiles suggesting that a number of mechanisms may be operating in the epididymis that bring about post-translational modifications to the sperm surface. In order to study the precise mechanism(s) involved in this process, it was absolutely essential to recover specific proteins identified on the polyacrylamide gel. Since no apparatus for this purpose was available commercially, it was thought worthwhile to fabricate one that could serve our need.

In this chapter, a novel system is described which is designed and fabricated for on line elution of protein from a column of polyacrylamide gel. Samples are loaded on this gel and proteins are resolved by electrophoresis. As the resolved proteins make their exit towards the bottom of the gel they are eluted in a stream of desired buffer.

5.2 MATERIALS AND METHODS

Principle

Since the purpose of designing and fabrication of the equipment for on line elution of proteins from polyacrylamide gels cast in

specially designed tubes is based on separation of proteins by polyacrylamide gel electrophoresis, the factors influencing the process would be similar to those of SDS-PAGE. The separation of proteins on SDS-PAGE is dependent on the charge/mass ratio of proteins when coupled with sodium dodecyl sulfate. Mobility of proteins on SDS-PAGE is also affected by the concentrations of polymers used, length of the gel and current.

The selection of the concentration of both monomers is of critical importance. T is a ratio of the total mass of both monomers to the volume of their solution and is expressed as a percentage. While C is the ratio of the mass of methylene bisacrylamide to that of both monomers, also expressed as a percentage. The variations of T lie within a range of 3% to 30% while in the case of C they range from 1% to 5%. The ratio in the present study has been kept at 30:1. The T and C values affect the migration of biopolymers in polyacrylamide gels. The breaking effect due to friction against the gel is expressed as the reduction of electrophoretic mobilities of charged molecules (u') compared to those in a liquid u_o (i.e. upon free boundary electrophoresis at the same pH and ionic strength).

Electrophoretic mobility is defined as the migration velocity of charged molecules in cm/h, referred to as 1V/cm. u_o is determined by the ratio of the net charge of the macromolecule (at a given pH) relative to its mass. The field strength induced on a molecule is proportional to its linear size. In general, the electrophoretic mobilities of the majority of acidic proteins (u_o)

at pH 8.8 fall in the range of 0.1 to 0.5 cm/h per 1 V/cm. The effective electrophoretic separation of proteins on polyacrylamide gels can be performed at a u': u_o ratio equal to 0.1 to 0.2. This effectively means that the electrophoretic mobilities of proteins in polyacrylamide gel covers an interval of 0.01 to 0.1cm/h per 1V/cm. At low potential of say 10-20 V/cm, the migration velocities will be in the range of 0.1-2 cm/h and for a 10cm gel the fastest moving proteins can reach its end in five hours, while the slowest moving proteins will have moved only 0.5cm from the point of application. For proteins of known molecular weights or proteins varying remarkedly from each other in size or charge, the run may be carried out under conditions enabling higher u' values, i.e. in gels of larger porosity, thus shortening the fractionation time by a factor of 2-3. The choice of T value depends on the nature of the difference in electrophoretic mobility, namely whether it is charge or size.

The migration of proteins in gels

The molecular weights of the great majority of individual proteins does not exceed 500,000, and agarose gels in general are unsuitable with a few exceptions. In general, 5% to 20% polyacryl amide gels are imminently suitable for separation of most proteins. The proteins are amphoteric and their net charge and consequently charge : mass ratio may be governed by alterations in the pH of the buffer used to prepare the gel and the reservoir buffers. The optimal pH value of a running buffer is that which ensures the maximum difference in charge of component proteins and

not the maximum charge as such. In general, extreme values of pH are used as they are quite far removed from isoelectric pH values of constituent proteins. For acidic proteins, the optimal pH values fall in the neutral or slightly alkaline region and such proteins migrate towards the anode. For basic proteins, it is best to use slightly acidic buffer. These proteins will migrate towards the cathode, differing from each other by net positive charge. The buffer molarity is kept in a range of 0.1M to 0.2M.

Electric field strength

The electrical resistance of a buffer is determined by two factors, namely by the concentration of free ions and by their electrophoretic mobility, the later being of greater importance. The electric current is the same throughout the length of a circuit i.e. at any cross section of a slab gel or tube gel, no drop or jump in current is physically possible. The voltage and electric field are, however, not constant and in gel comprising two resistance, the voltage drop will be a product of the current and the individual resistance and the total voltage distribution will be equal to the sum of individual voltage drop.

Running buffer

Complete protonation of Tris-HCl at pH 6.7 increases its conductivity to nearly 90% or more. This will be nearly 0.1M with respect to Cl^-. The Tris-HCl buffers of pH 8.9 have a conductivity less than that at pH 8.1 due to the lower Cl^- ion and Tris H^+ ion concentrations.

Heat evolution

The electric power which is dissipated in the gel as heat is equal to the product of the current and voltage drop in the gel. Use of voltage above 200 V/cm for a 10cm long gel will lead to distortion of bands. Literature reports indicate air cooled systems much more effective than the conventional cooling systems. The use of a cold room has been favoured in the current study.

The above mentioned parameters were taken into consideration while designing, fabricating and developing the apparatus for the simultaneous elution of proteins separated during an electrophoretic run.

5.2.1 Basic Components

The basic requirements for the implementation of the method are a power pack, the self fabricated apparatus, a peristaltic pump and a fraction collector. Of these basic components, the specifications of only the apparatus that was designed and developed in this laboratory are described.

The apparatus resembles a typical disc electrophoresis assembly in design. The key to the separation and elution of proteins lies in the specially fabricated gel tube. In principle, proteins are resolved in this tube by electrophoresis and eluted from the bottom in the order in which they make their exit. Electrophoresis is carried out at desired current/voltage using a power pack (PS1200, Hoefer Scientific Instruments, U.S.A.). The flow rate of elution is controlled using a peristaltic pump

(Meclins, India) and the fractions emerging from the gel tube are collected in a fraction collector (Sisco, India) (Fig. 5.1).

5.2.2 Design and Fabrication of the Apparatus

Perspex sheets (0.5cm thickness) are cut to size and used in all the fabrications. The apparatus consists of two chambers (each 8 x 8 x 5cm) that can hold 240ml of buffer. But in practice, only 200ml of buffer is used. The lower chamber supports the upper chamber at the four corners by pegs (12cm long). Since the upper and lower chambers are meant to be reservoirs for buffer, they are leak proof. The distance between the two chambers is adjusted to accomodate a single large gel tube. This gel tube is inserted through the rubber grommet at the bottom of the upper chamber on one end, and dips into the buffer of the lower chamber at the distal end. The anode contacts the buffer from the base of the lower chamber and the cathode dips into the buffer from the lid of the upper chamber. Each electrode is made of platinum wire (0.8mm thick) bound around a circular perspex ring. Care is taken to ensure that the two electrodes and the gel tube are aligned in one axis (Fig. 5.2).

5.2.3 Fabrication of Gel Tube

A tube made of Corning glass (1.5cm inner diameter) is cut to a length of 15cm. This tube holds the gel and forms a bridge between the upper and lower electrophoretic chambers. To this main tube, perpendicular to the long axis and 2cm from the bottom, two glass tubings (4cm length and 0.1cm internal diameter) are fused like side arms, exactly opposite each other. These tubes

serve as inlet and outlet to the elution buffer. Elution buffer from a reservoir is connected to the inlet. The outlet is linked with a fraction collector through a peristaltic pump using a teflon tubing.

5.2.4 Casting the Gel

Reagents : All the reagents are prepared as described earlier in
Section 4.2.3.

(a) Gel plug

Composition of gel plug is given below

Reagents	Volume (ml)
Acrylamide: Bisacrylamide (30:0.8)	6.9
0.5M Tris-HCl buffer pH 6.8	2.5
1.5% Ammonium persulfate	0.5
10% Sodium dodecyl sulfate	0.1
TEMED	0.008

(b) Resolving gel

The composition of resolving gel for low (5%) and high (10%) SDS-Polyacrylamide gel is given below.

Reagents	Volume (ml)	
	5%	10%
Acrylamide: Bisacrylamide (30:0.8)	5	10
3.0M Tris-HCl buffer (pH 8.8)	3.75	3.75
1.5% Ammonium persulfate	1.5	1.5
10% Sodium dodecyl sulfate	0.3	0.3
Water	19.45	14.45
TEMED	0.015	0.015

(c) Stacking gel

The composition of stacking gel is as follows.

Reagents	Volume (ml)
Acrylamide : Bisacrylamide (30:0.8)	2.5
0.5M Tris-HCl buffer (pH 6.8)	5.0
1.5% Ammonium persulfate	1.0
10% Sodium dodecyl sulfate	0.2
Water	11.3
TEMED	0.015

Elution buffer: 0.125M Tris-HCl buffer (pH 6.8) containing 0.1%
Sodium dodecyl sulfate.

Procedure

The lower end of the fabricated gel tube is first sealed with
parafilm and held in an upright position in the special apparatus
designed for electrophoresis and elution. The two side arms
comprising the inlet and outlet are connected to the reservoir and
fraction collector, respectively. A 2.0cm thick 20%
polyacrylamide gel is poured, such that a plug is formed above the
parafilm, the top layer of which is in the line with the side
arms. This is then overlayered with water. Once the gel has
polymerized the water is drained and the lower gel plug is topped
by a 1mm thick layer of 50% sucrose through the inlet and care is
taken that no air bubbles are trapped in any part of the assembly
from the reservoir to the outlet point at the fraction collection
end.

On top of the sucrose layer a 10cm long resolving gel of desired concentration is carefully layered. Care is taken that no gel solution enters the inlet or outlet, where polymerization of gel would mean that the flow of buffer would be obstructed. The gel is once again overlayered by water which is aspirated once the resolving gel has polymerized. The resolving gel is covered with a 1.0cm stacking gel. The gel mixture is again layered with water which is drained out after the gel has polymerized. At this stage the sucrose in the eluting space, the inlet and outlet is replaced with 0.125M Tris-HCl (pH 6.8) containing 0.1% SDS by continuously flushing this solution through the inlet and draining it out of the outlet. Incidentally, this solution is also used as the elution buffer. The parafilm at the lower end of the gel tube is removed and the gel tube is ready for use.

5.2.5 Preparation of Sample

For standardization of the procedure, standard proteins viz. lysozyme (Mr 14,300), carbonic anhydrase (Mr 29,000) and ovalbumin (Mr 45,000) from Sigma have been loaded as an aqueous preparation.

The procedure was then adapted for solubilized proteins from sperm membrane preparation. Generally when polyacrylamide gels are used, it is known that low molecular weight proteins resolve better at higher gel concentration and high molecular weight proteins separate satisfactorily on low concentration gels. Since sperm membrane proteins contained a mixture of both high and low molecular weight components, these two major components were preliminarily separated on a preparative polyacrylamide gel so

that the appropriate concentration of acrylamide can be used.

5.2.6 Preparative Gel Electrophoresis

The solubilized sperm membrane sample in 0.0625M Tris-HCl buffer (pH 6.8), 2.0% SDS, 5% β mercaptoethanol and 20% glycerol are initially subjected to electrophoresis on preparative 10%, polyacrylamide slab gels. The preparative gel comprises a 15cm wide, 16 cm long and 0.15cm thick slab gel. The comb inserted into the stacking gel has two wells one 12 x 1.5 x 0.3cm and another 0.6 x 1.5 x 0.3cm. The gel is run at a constant current of 30mA. Once the tracking dye has moved 10cm into the resolving gel, the current is switched off and the gel plate on one side is carefully lifted. The lane below the smaller well is subjected to side strip staining. Desired portion of the gel containing either low or high molecular weight components are identified by this strip staining method.

5.2.6.1 Side strip staining

1. After running the gel, one of the plates is removed and a straight edge or ruler is placed on the gel so that it runs vertically up the gel about 1cm from one edge.

2. With a small cork borer a series of plugs about 4-5mm in diameter are removed. The plugs are removed at random about 10 holes in a line.

3. A vertical line dissecting the holes is cut. The side strip is removed and stained by the method of Lee et al., (1987).

4. The guide holes are used to line up the gel with the rest of the gel.

5. Low and high molecular weight containing portions of the gel are separated for loading on to the electrophoretic assembly coupled with on line elution of the separated proteins.

Copper staining of gels (Lee et al., 1987)

A useful alternative to the more traditional staining techniques of Coomassie brilliant blue or silver staining is copper staining. The incubation of gels in a solution of copper chloride allows the formation of a white opaque precipitate apparently involving both Tris and SDS. Protein bands remain clear, leaving a negative image of the polypeptide separation pattern. The proteins are not fixed in the gel and can be eluted by simple removal of the copper ions by chelation with EDTA.

1. After the side strips have been removed from the parent gel, they are washed briefly with distilled water consisting of several changes over 30 seconds. Longer washes will allow Tris or SDS to elute and are avoided.

2. The gel is placed in a tray (glass or plastic) and atleast five gel volumes of 0.3M $CuCl_2$ is added.

3. The gel is incubated in this solution with agitation for 5 minutes. As the $CuCl_2$ enters the gel, a white precipitate is formed in the regions of the gel that do not contain proteins.

4. The gel is washed for several minutes with distilled water and viewed against a dark background.

Sensitivity is approximately 1µg per band for 1mm thick gels. The macerated gel slices from desired gel sections containing high/low molecular weight proteins are loaded on the gel tube.

5.2.7 Optimization of operational conditions

Any equipment functions effectively only when critical parameters are optimized. In the present case, two events are critical

a)   the rate of electrophoretic separation and

b)   the rate of elution.

In order to choose the ideal conditions, trials are performed with the apparatus using three low molecular weight marker proteins at 10% polyacrylamide gel concentration. The electroelution profile of a mixture of lysozyme, carbonic anhydrase and ovalbumin are monitored at:

a)   30mA constant current and elution flow rate of 2ml/5min.

b)   30mA and elution flow rate of 1ml/min.

c)   20mA and 2ml/5min.

d)   20mA and 1ml/1min.

Since a current of 20mA and an elution flow rate of 1ml/min are found to be most suitable, same conditions are used while dealing with solubilized sperm membrane proteins.

5.3   RESULTS

The design of the apparatus is such that simultaneous elution of proteins is possible during electrophoresis. When a mixture of commercially available low molecular weight standards, lysozyme, carbonic anhydrase and ovalbumin are used as samples and 2ml

fractions are collected, three distinct peaks are observed in the elution profile.

Initial experiments with these low molecular weight protein markers helped in establishing the ideal conditions required for best resolution. After trying out a number of runs with variations in current applied to the polyacrylamide columns and different flow rates, certain facts came to light. It became apparent that for best results current applied should be well balanced. Data obtained on runs carried out at 30mA and 20mA with flow rates of 2ml/5min and 1ml/min are presented with the view to emphasize this fact (Figs. 5.4 - 5.7).

The salient observations from the elution profiles are highlighted as follows:

At a constant current of 30mA and 2ml/5min flow rate, the three low molecular weight standards lysozyme (Fraction number 15 - 25), carbonic anhydrase (Fraction number 30 - 45) and ovalbumin (Fraction number 70 - 80) are well resolved. However, if the flow rate is increased to 1ml/min the elution of lysozyme (Fraction number 17 - 20) carbonic anhydrase (Fraction number 34 - 40) and avalbumin (Fraction number 70 - 75) is delayed. At a constant current of 20mA the picture that emerges is almost similar. The elution pattern of lysozyme (Fraction number 20 - 26), carbonic anhydrase (Fraction number 45 - 55) and ovalbumin (Fraction number 108 - 115) at a flow rate of 2ml/5min is changed to lysozyme (Fraction number 23 - 27), carbonic anhydrase (Fraction number 48 - 52), and ovalbumin (Fraction number 112 - 116) when flow rate is

increased to 1ml/min. Thus, in both instances, a change in flow rate from 2ml/5min to 1ml/min results in a reduced band width and increased peak height of the eluting components. Both these features are indices of better resolution.

When the same results are reviewed from a different angle some interesting facts emerge. At a constant flow rate of 2ml/5min with the current applied is 30 mA, the elution profile obtained is lysozyme (Fraction number 15 - 25), carbonic anhydrase (Fraction number 30 - 45) and ovalbumin (Fraction number 70 - 80). On the other hand, reducing the current to 20mA and keeping the flow rate of 2ml/5min, delays the appearance of lysozyme (Fraction number 20 - 26), carbonic anhydrase (Fraction number 45 - 55) and ovalbumin (Fraction number 108 - 115) peaks.

When the flow rate is kept constant at 1ml/min the elution profiles of the standard proteins at 30mA and 20mA are lysozyme (17-20), carbonic anhydrase (34-40) and ovalbumin (70-75), and lysozyme (23-27), carbonic anhydrase (48-52) and ovalbumin (112-116) respectively. The message that these results convey is that a current of 20mA for electrophoresis and a flow rate of 1ml/min is most suitable for the operation of the apparatus that was designed. Under these conditions, proteins that emerged from the gel column during electrophoresis eluted into the buffer and did not penetrate the gel plug. With the apparatus having passed the operation test, it was important to know if the apparatus can accomodate a different mode of sample application. It must be remembered that the low molecular weight standards used in the

optimization experiment are aqueous preparations. Since the ultimate objective is to use this technique for resolving and isolating sperm membrane proteins which are entrapped in gels, minced gel containing the proteins of interest are directly loaded. Two categories of sample are loaded.

(a) Gel mince containing low molecular weight proteins and

(b) Gel mince containing high molecular weight proteins.

Low molecular weight proteins are loaded on a 10% polyacrylamide column, while high molecular weight proteins are loaded on 5% polyacrylamide columns; the elution profiles obtained are presented in Figs. 5.8 and 5.9. It appears that there is 100% recovery of the proteins loaded since Coomassie brillant blue staining of both the 10% and 5% polyacrylamide column did not detect the presence of any proteins in them (Fig. 5.10 a,b). Moreover, several proteins are resolved as seen from distinct peaks in the elution profile. The homogeneity of the protein in samples collected from each peak is confirmed by single bands obtained on SDS-PAGE (Figs. 5.11 and 5.12).

5.4 DISCUSSION

Based on some sound theoretical concepts, an apparatus is developed for the simultaneous elution of proteins during an electrophoretic run. The assembly is an improved version of a disc electrophoretic apparatus tailored to meet the requirements of resolving and isolating desired proteins from a complex mixture.

Fig. 5.1　Basic Components of the Assembly for On-Line Elution of Proteins During Electrophoresis. (A) Powerpack, (B) Reservoir for elution buffer, (C) Apparatus for On-line elution of proteins, (D) Peristaltic pump and (E) Fraction collector.
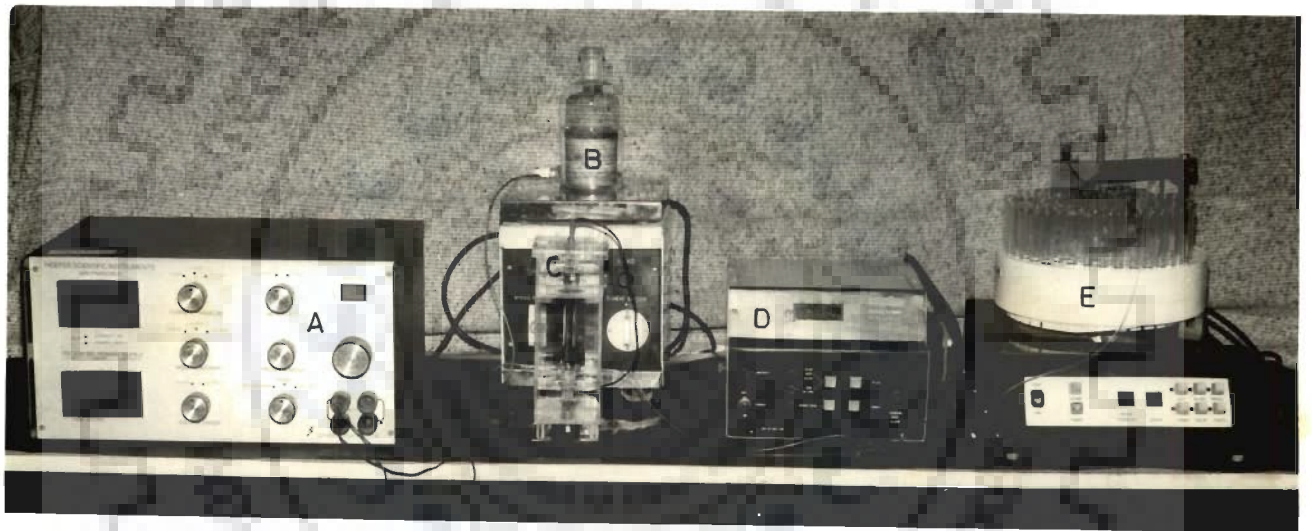
FIG. 5·1

Fig. 5.2   The Apparatus for On-Line Elution of Proteins During
           Electrophoresis. (A) Terminals,   (B) Lid, (C) Upper
           buffer chamber, (D) Cathode, (E) Pegs, (F) Elution
           tubing, (G) Outlet for elution buffer, (H) Inlet for
           elution buffer, (I) Anode and (J) Lower buffer chamber.

FIG. 5·2

SAMPLE

STACKING GEL

ELUTION BUFFER
0.125 M TRIS HCl (pH 6.8)
0.1 % SDS

RESOLVING GEL

INLET

0.125M TRIS HCl BUFFER (pH 6.8); 0.1 SDS

OUTLET

LOWER GEL PLUG

FRACTION
COLLECTOR
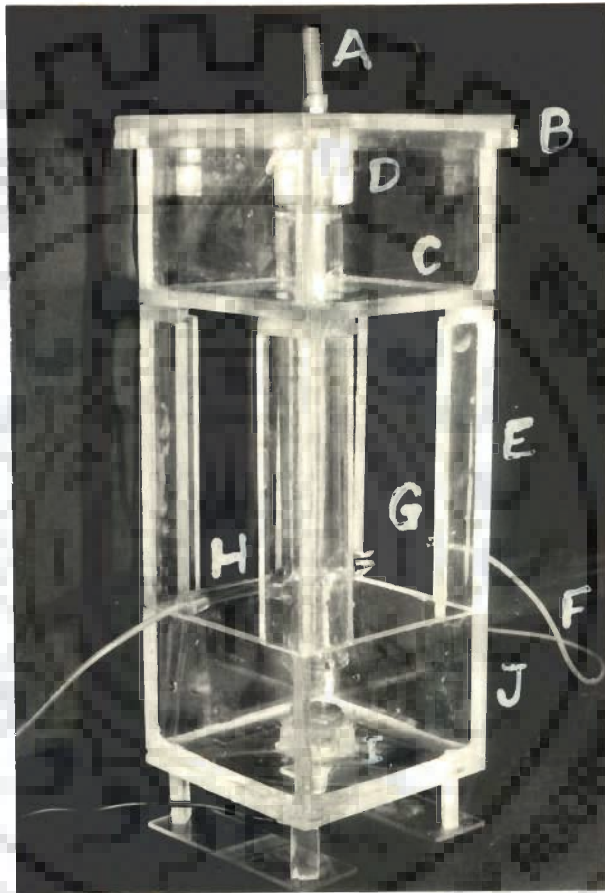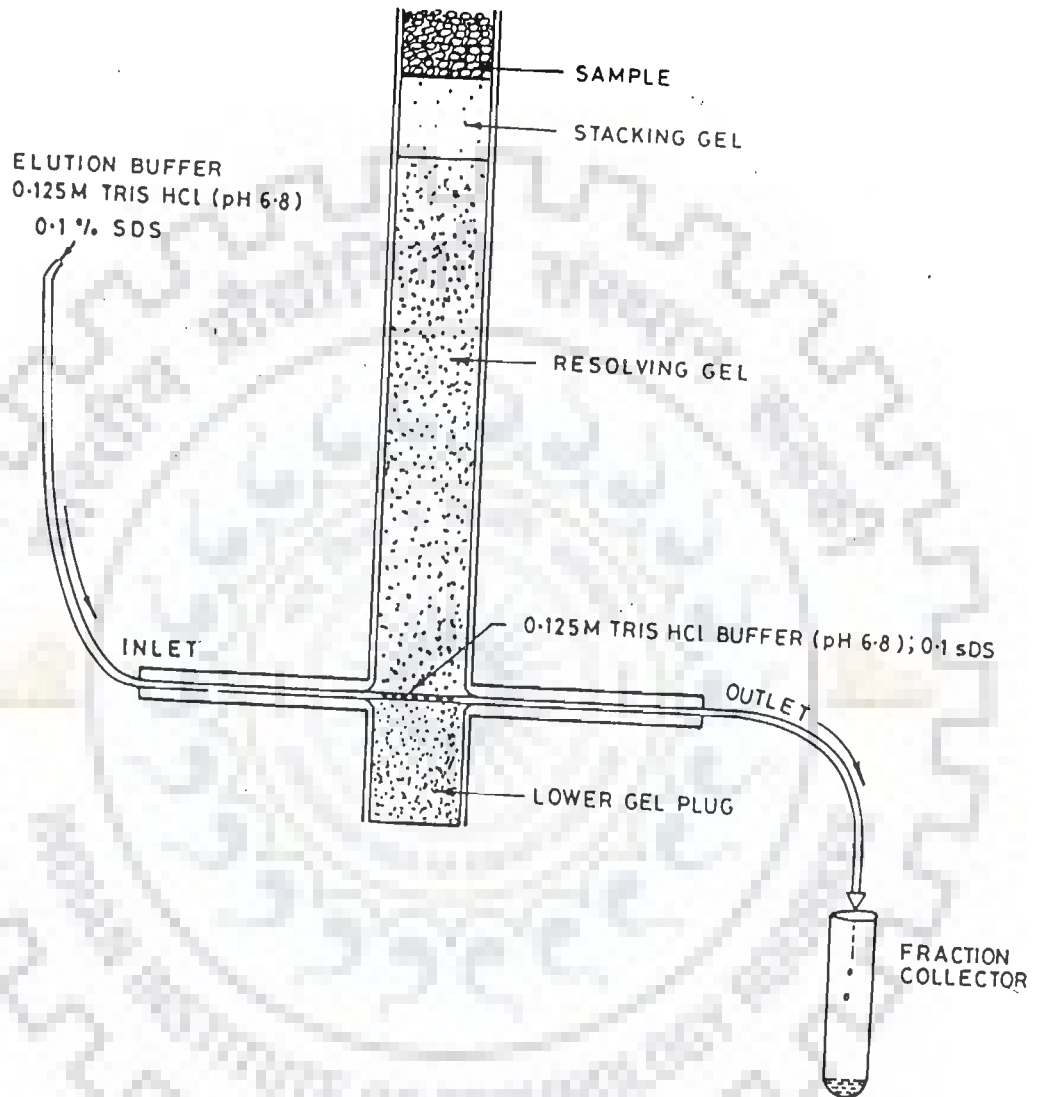
FIG. 5.3—SCHEMATIC DIAGRAM OF THE ELECTROELUTION TUBING

Fig. 5.4   The Elution Profile of Standard Low Molecular Weight
Proteins Electrophoresed on a 10% SDS-PAGE Column at 30
mA Constant Current.   Standard low molecular weight
proteins Lysozyme (14.4 kD), Carbonic Anhydrase (29 kD)
and Ovalbumin (45 kD) are applied   and eluted at a flow
rate of 2 ml/5 min.
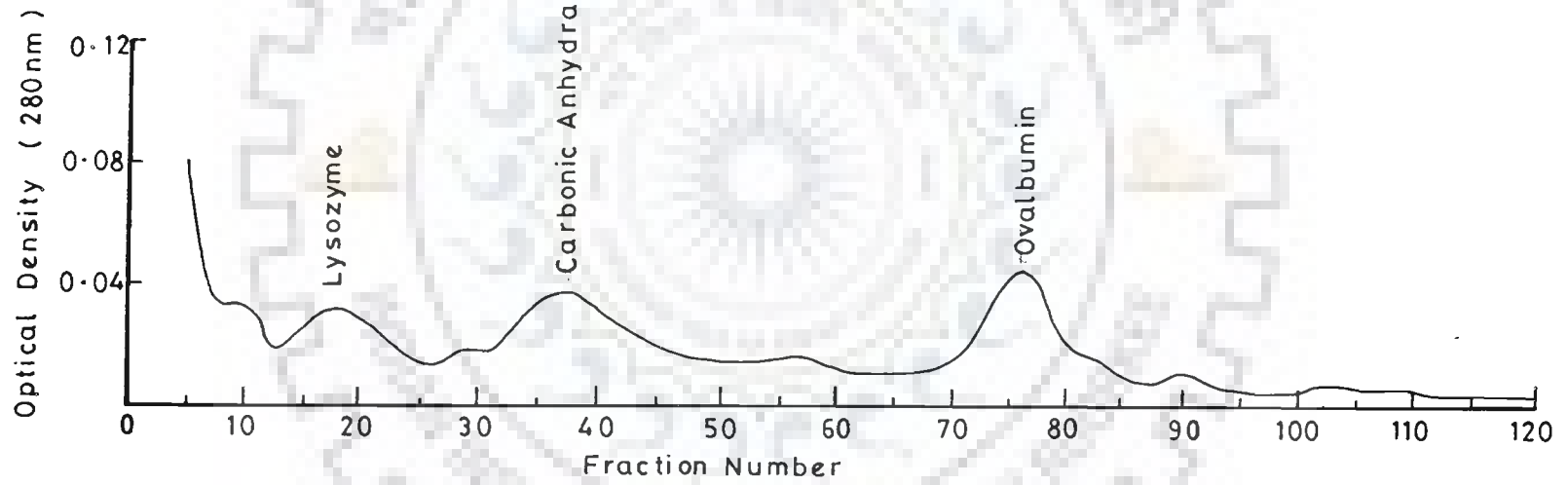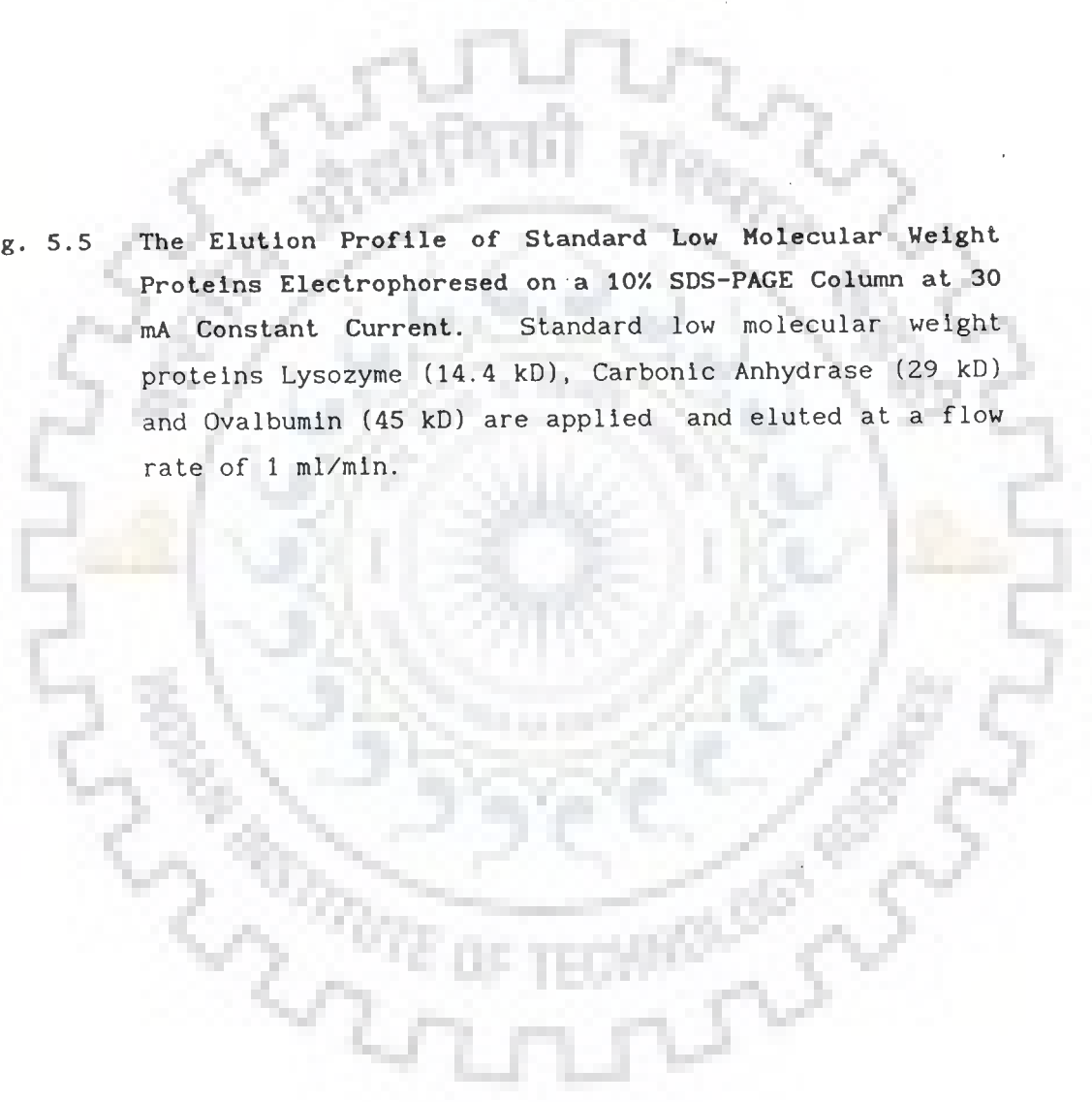
FIG. 5·4

Fig. 5.5 The Elution Profile of Standard Low Molecular Weight
Proteins Electrophoresed on a 10% SDS-PAGE Column at 30
mA Constant Current.   Standard low molecular weight
proteins Lysozyme (14.4 kD), Carbonic Anhydrase (29 kD)
and Ovalbumin (45 kD) are applied and eluted at a flow
rate of 1 ml/min.
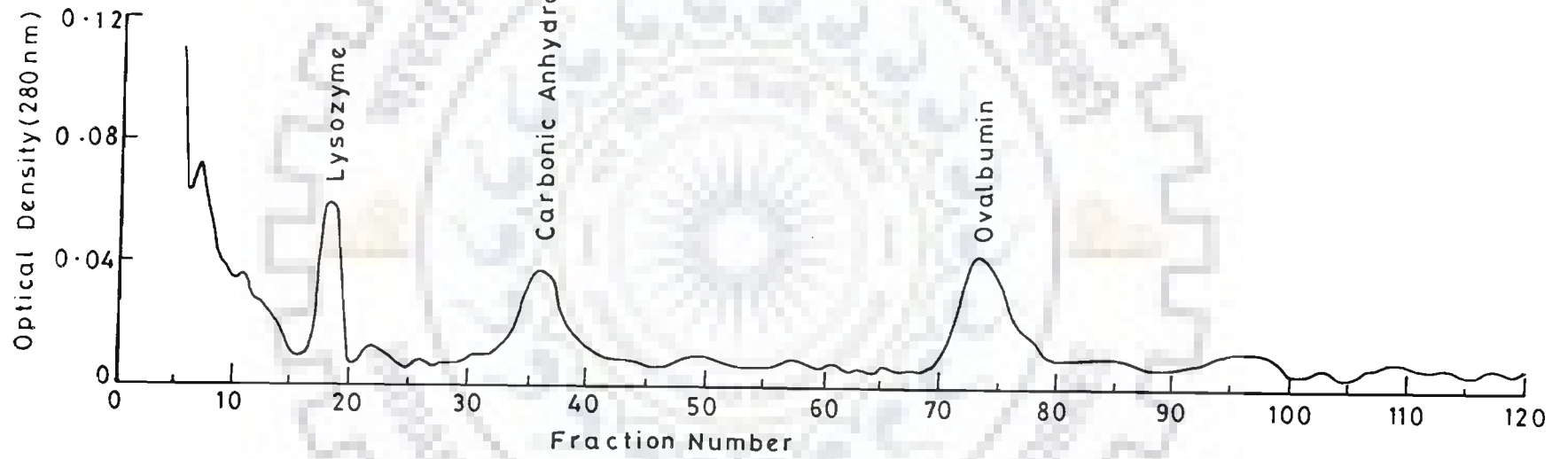
FIG. 5·5

Fig. 5.6   The Elution Profile of Standard Low Molecular Weight
Proteins Electrophoresed on a 10% SDS-PAGE Column at 20
mA Constant Current.   Standard low molecular weight
proteins Lysozyme (14.4 kD), Carbonic Anhydrase (29 kD)
and Ovalbumin (45 kD) are applied and eluted at a flow
rate of 2 ml/5 min.
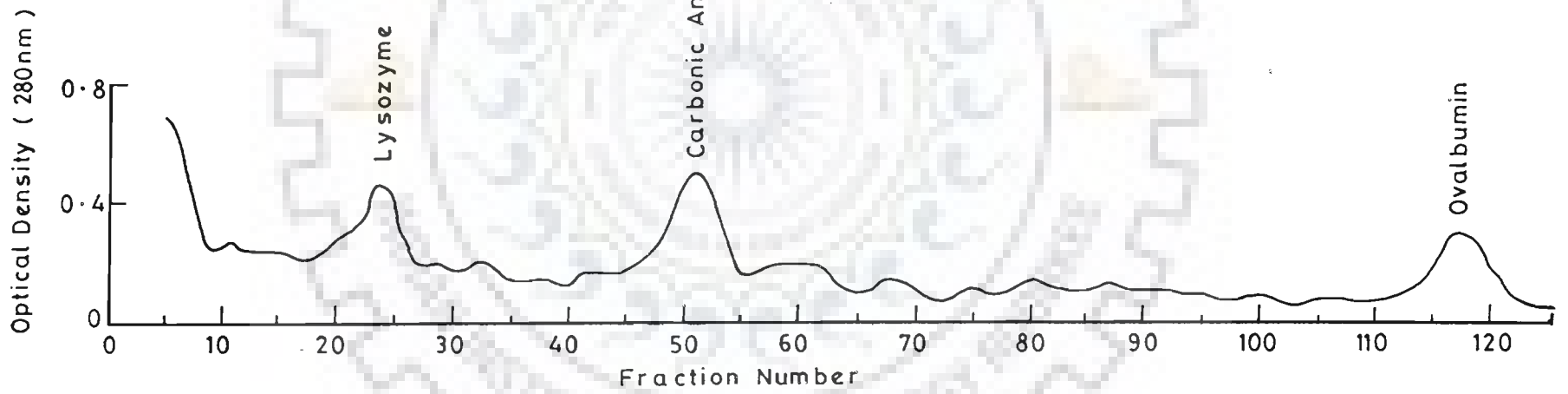
FIG. 5·6

Fig. 5.7 The Elution Profile of Standard Low Molecular Weight
Proteins Electrophoresed on a 10% SDS-PAGE Column at 20
mA Constant Current.   Standard low molecular weight
proteins Lysozyme (14.4 kD), Carbonic Anhydrase (29 kD)
and Ovalbumin (45 kD) are applied and eluted at a flow
rate of 1 ml/min.
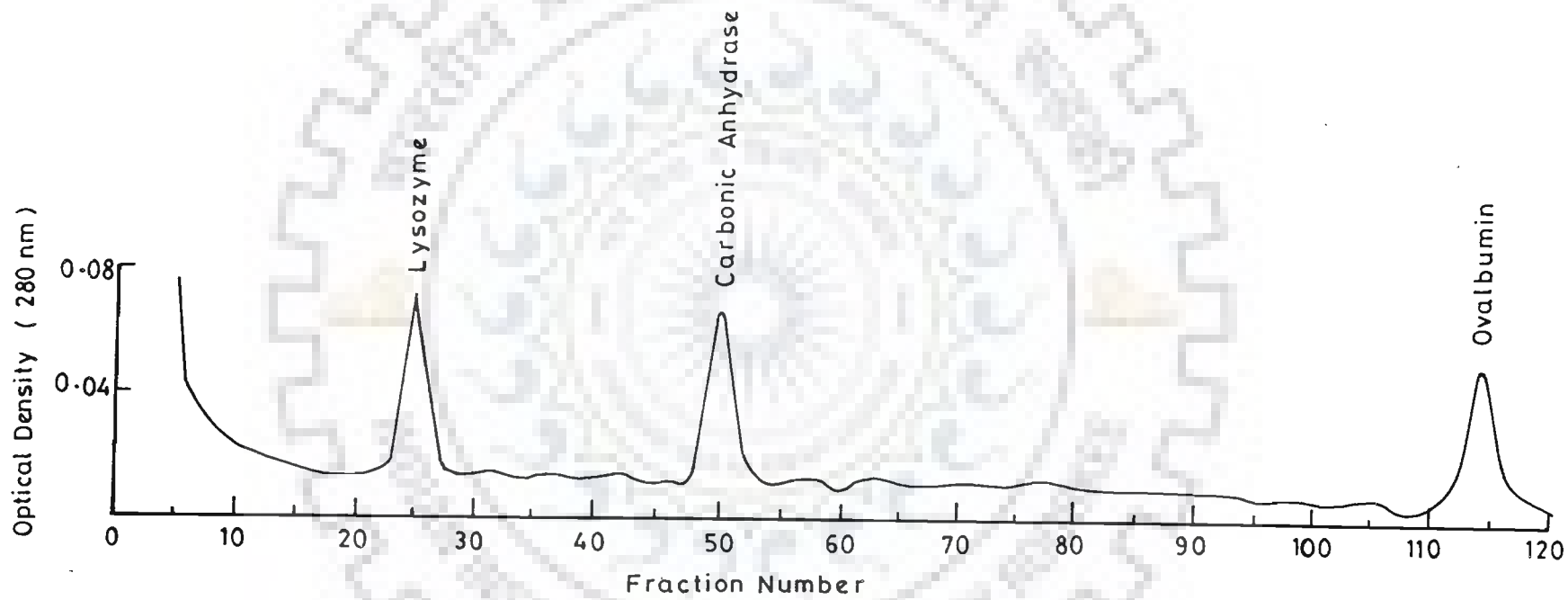
FIG. 5·7

Fig. 5.8   The Elution Profile of Low Molecular Weight Sperm
           Membrane   Proteins   separated   by   the   on-line
           electroelution on 10% SDS-PAGE column electrophoresed at
           20 mA constant current and eluted at a flow rate of
           1 ml/min.
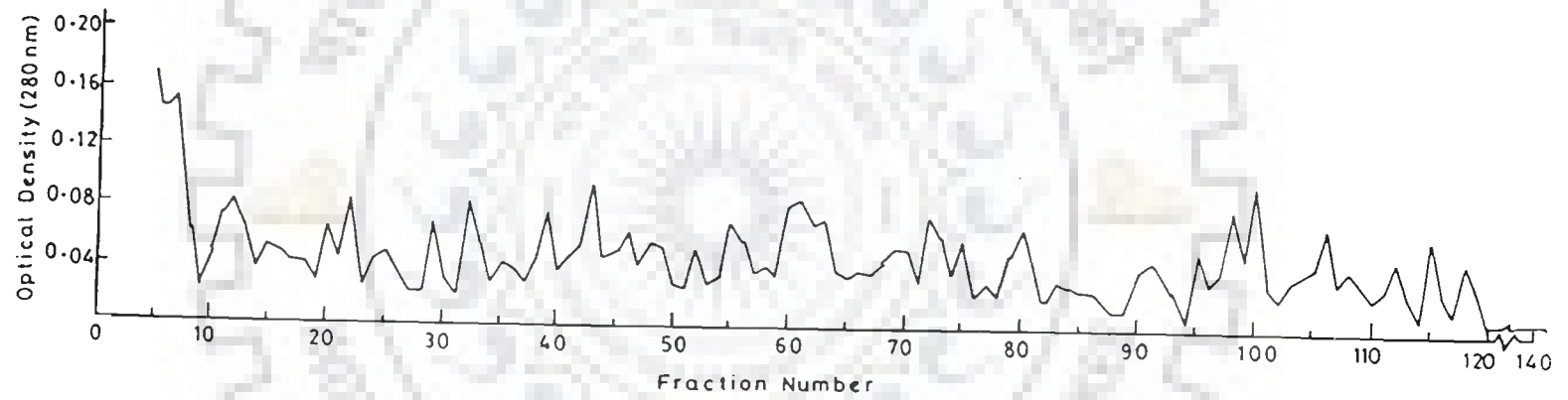
FIG. 5·8

Fig. 5.9   The Elution Profile of High Molecular Weight Epididymal
           Sperm Proteins separated by on-line electroelution on 5%
           SDS-PAGE column electrophoresed at 20 mA constant
           current and eluted at a flow rate of 1 ml/min.
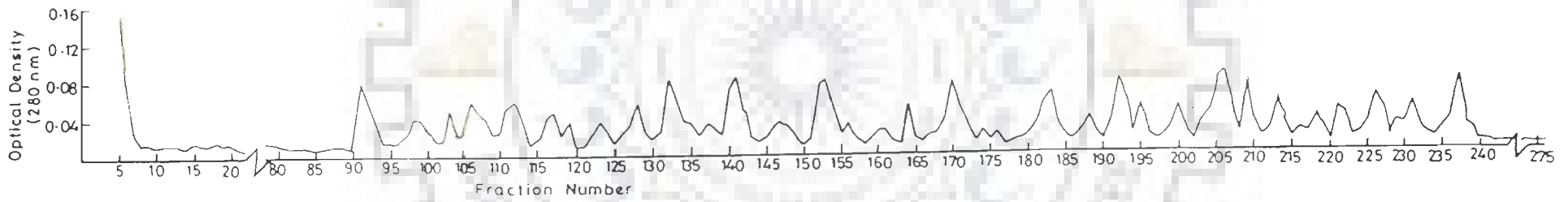
FIG 5·9

Fig. 5.10 SDS-PAGE Columns Recovered From the Gel Tube After Completion of On-Line Elution. (a) 10% SDS-PAGE columns recovered after electroelution of low molecular weight proteins and (b) 5% SDS-PAGE column recovered after electroelution of high molecular weight proteins when stained with Coomassie brilliant blue show the absence of any protein.

(a)   (b)

FIG. 5·10

Fig. 5.11 Electrophoretogram of the Low Molecular Weight Protein
Fractions of Epididymal Sperm Membranes on 10% SDS-PAGE
obtained after On-line elution on 10% SDS-PAGE.

Fig. 5.12 Electrophoretogram of the High Molecular Weight Protein
Fractions of Epididymal Sperm Membranes on 10% SDS-PAGE
obtained after On-line elution on 10% SDS-PAGE.

Fraction No.   118 106 96 91 80 72 43 32 22 12 10
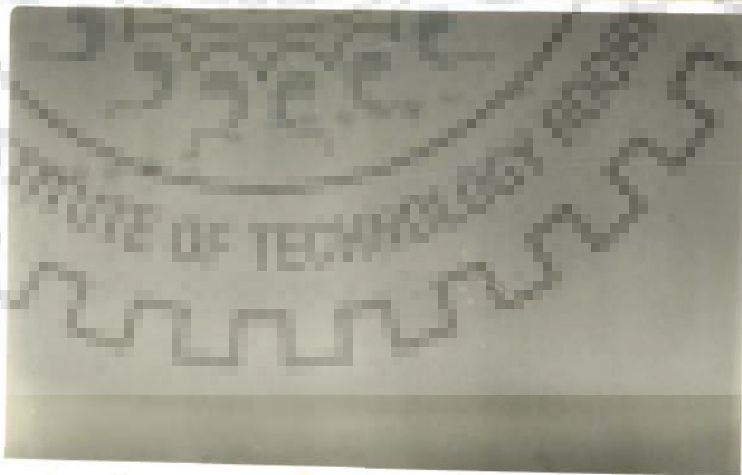
Fraction No. 91 98 106 112 117 131 141 153 170 183 192 200 206 209 213 215 220 225 231 237

Since electrophoresis is the basis of protein separation, not much effort was is needed for tackling problems associated with this aspect of the technology. The principle involved in the choice of buffers (McLellan, 1982), the length of resolving gel (Osterman, 1984 and Hames and Rickwood, 1990), the need for stacking gel (Davies, 1964 and Ornstein, 1964), the utility of varying gel concentration (Dunker and Rueckert, 1969 and Fehrnstrom and Moberg, 1977) and current to be employed are known (Osterman, 1984). Therefore, appropriate conditions are chosen for obtaining best resolution as far as proteins are concerned.

The actual problem arises when electrophoresis is combined with elution. Not much has been done in this area and therefore optimization is required at this stage. The speed of electrophoresis is to be matched with the speed of elution in order to get the best from the apparatus. This is easily achieved using proteins of known molecular weight and different electrophoretic mobilities.

As is evident from the results, if the current applied is large, the rate of protein movement through the gel is fast. If this is not matched with an appropriate flow rate of elution, there is a tendency for the resolved proteins to get into the gel plug. To avoid this, if the flow rate of elution is enhanced, then the resolution of proteins is lost. Application of a small current slows down the rate of movement of protein through the polyacrylamide gel. The separation of protein is good but the flow rate of the elution buffer has to be carefully regulated. If

the flow rate is too high, there will be unnecessary dilution of the resolved protein. On the other hand, if flow rate is too low then there is a tendency for mixing of proteins that are resolved by the polyacrylamide gel. Hence, the current applied to the gel and the rate of elution are critical factors that have to be effectively balanced.

The present investigation clearly demonstrates that the aqueous preparations, as well as gels that have protein entrapped in them could be mixed and used as sample. The mechanism of operation involved in the apparatus seems adequate to resolve these entrapped proteins on a column of polyacrylamide which is simultaneously eluted. For biological samples, it is advisable to broadly separate the components into high molecular weight and low molecular weight fractions before applying the same on the designed apparatus. Low molecular weight proteins could be resolved on 10% polyacrylamide while high molecular weight proteins can be separated on a 5% polyacrylamide column.

One particularly striking feature is the 100% recovery of proteins from gels. This is evident from the fact that the column of polyacrylamide does not stain with Coomassie blue after the electrophoretic run. In addition, since the protein of interest could be fully recovered with and the procedure being non-distinctive, it might be used for preparative purposes too.

The apparatus offers several advantages over conventional technologies using chromatography. The procedure involved is simple and reproducible. If the current used for electrophoresis

and the flow rate of elution are fixed, then it appears the method could also be calibrated for determining the molecular weight of the eluted proteins.
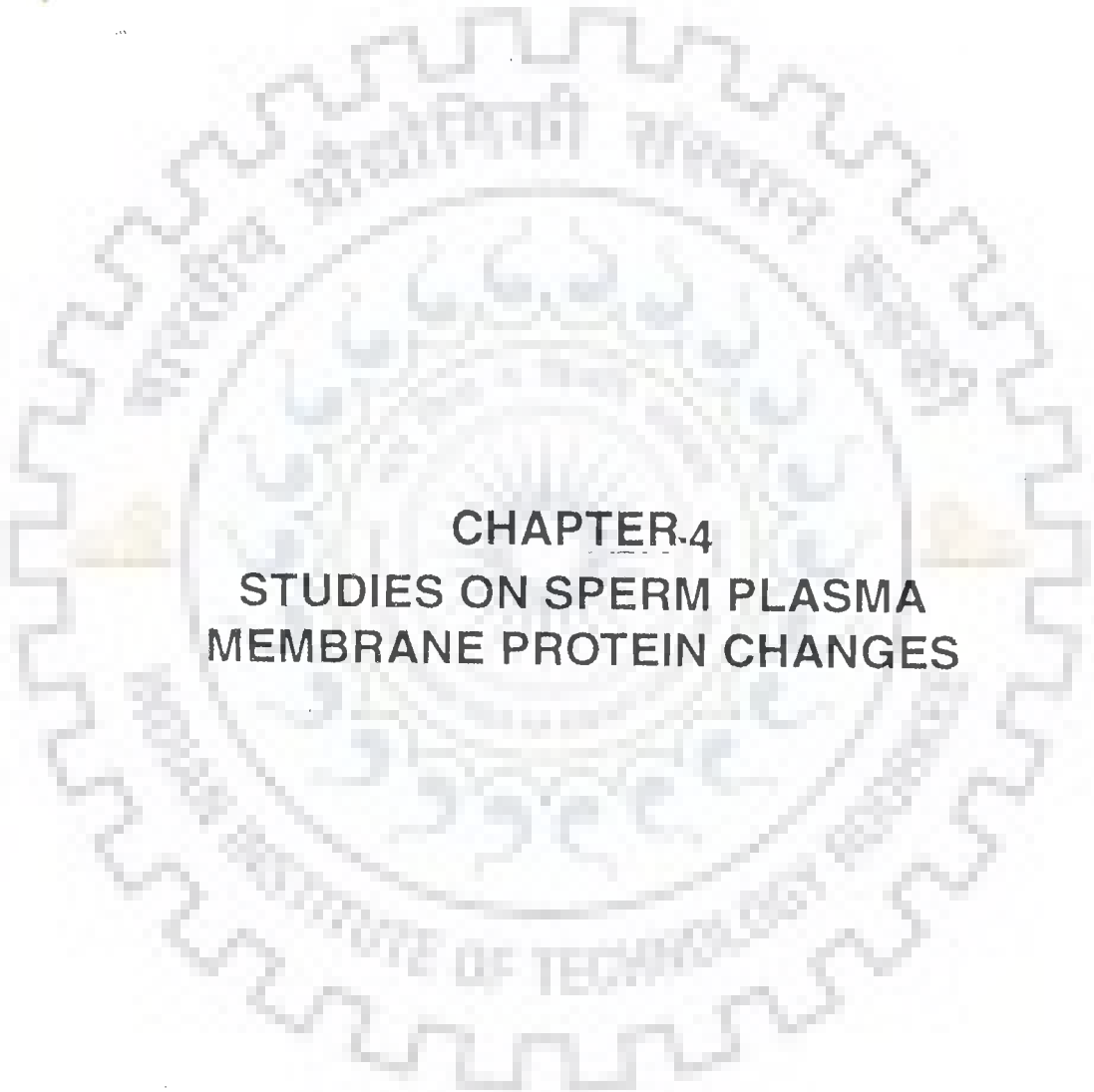
One of the aspects of the apparatus designed is the tremendous potential for automation. In modern times, automation is a convenience that is much sought after. Once the conditions have been standardized for a particular separation, then simple attachments like an appropriate detector and a recording system would complete the automation.

5.5 CONCLUDING REMARKS

1. A system has been developed for the simultaneous elution of proteins from polyacrylamide during an electrophoretic run.

2. The assembly resembles a disc gel electrophoretic apparatus with a single gel tube which is rather large sized and has provisions for elution with desired buffer towards the bottom.

3. A gel of 1.5cm diameter, electrophoresed at a constant current of 20mA and eluted at a flow rate of 1ml/min is found to be ideal to obtain best resolution.

4. The apparatus could also be effectively used for solubilized protein of sperm plasma membrane.

5. By varying the concentration of polyacrylamide the same apparatus can be used to separate and isolate proteins of both low and high molecular weight.

6. The resolution of proteins not only depends on the concentration of polyacrylamide but also on the flow rate of elution.

7. The separation is reliable, reproducible and particularly useful for proteins with differences in charge and molecular weight.

8. From the technical point of view the procedure for separation and isolation of protein is simple, time saving, cost effective, non-distinctive and recovery of protein of interest is almost cent percent.

CHAPTER-4
STUDIES ON SPERM PLASMA MEMBRANE PROTEIN CHANGES

6.1   INTRODUCTION

There seems little doubt that proteins are important constituents of the sperm membranes, essential for fulfiling the objective of fertilizing the ova.   A number of proteins have been identified on the surface of mammalian sperm that have a crucial role to play in processes such as acquisition of forward motility (Acott and Hoskins, 1978), flagellar straightening (Cornwall and Chang, 1990), regionalization of sperm surface (Bearer and Friend, 1982, Blobel et al., 1990 and Phelps et al., 1990) and sperm-egg interaction (Naz, 1990).   It thus appears, that sperm must acquire and display the correct array of proteins on their surface in order to successfully interact with ova.

It has been clearly demonstrated that the membrane proteins of testicular sperm are not adequate for this purpose, and extensive remodelling of the sperm surface must occur.   This primary requirement is met within the epididymis where sperm surface transformations are brought about.   The elaborate enzymatic machinery together with the secretory and absorptive nature of the epididymal epithelium provides the right microenvironment for the modification of the sperm surface (Orgebin- Crist et al.; 1975; Eddy, 1988 and Jones, 1989b).   Obviously, several mechanisms are involved in changing the protein profiles of the sperm surface (Voglmayr et al., 1980).   They include

i)   Loss of sperm surface protein components,

ii)   Addition of new surface protein components and

iii) Processing of preexisting sperm surface protein components.

The current chapter focuses attention on the changing sperm surface protein profiles during passage through the epididymis. The aim is to analyze membrane proteins solubilized by SDS from epididymal sperm collected at various sites along the epididymal duct. From the preliminary data, proteins of interest have been isolated and subjected to peptide mapping. Further, immunological studies throw light on the mechanism by which sperm surface transformation is affected in the goat epididymis.

6.2 MATERIALS AND METHODS

Sperm is collected from goat epididymal tissue slices at three sites: the caput, corpus and cauda. Sperm plasma membranes are isolated by multiple centrifugation steps as described earlier (Section 3.2.2). Proteins from the membranes are solubilized with SDS and used for investigations.

Analysis of sperm membrane protein by SDS-PAGE alone is highly complicated and tedious because of the tremendous diversity in the nature of proteins involved. Besides, the recovery of proteins from these gels is often poor and unsatisfactory. This calls for more refined techniques to analyse proteins in detail. In the preceding chapter, it has been demonstrated that low molecular weight proteins are better resolved on 10% polyacrylamide gels. On the other hand, good separation of high molecular weight proteins is achieved on 5% polyacrylamide gels. To take the best advantage of this principle, an apparatus is designed for the on line elution of electrophoresed proteins. After initial separation of sperm membrane protein on 10% SDS polyacrylamide

slab gels, the portion of gels harbouring high and low molecular weight proteins are macerated separately and loaded on this self designed apparatus. The resolved proteins are eluted into buffer. The fraction containing the proteins are identified by measuring the optical density and then used for further studies.

6.2.1 Separation of High and Low Molecular Weight Sperm Membrane Proteins

6.2.1.1 Preparation of sample

Membrane preparations from caput, corpus and cauda epididymal spermatozoa are used as the starting material. Membrane preparations containing 5mg protein are is solubilized in Laemmli's sample buffer containing 0.0625M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. The mixture is boiled for 3 minutes and cooled. The solubilized proteins are recovered after centrifugation.

6.2.1.2 Casting the gel

A 14 x 16 x 0.3cm, 10% Sodium dodecyl sulfate polyacrylamide gel has been used for preparative gel electrophoresis.

A gel with dimension of 14 x 16 x 0.3cm is cast. A comb that makes a single large well is used. The solubilized sperm membrane sample is loaded and run at 20 mA in the stacking gel and increased to 30 mA in the resolving gel. Once the tracking dye reaches a distance of 10 cm in the resolving gel, the run is discontinued. The glass plate on one side is gently lifted and

Composition of reagents for the preparation of 10% SDS-PAGE gel

Reagents	Stacking	Resolving
Acrylamide-Bisacrylamide (30:0.8)	2.5 ml	10 ml
Resolving gel buffer	-	3.75 ml
Stacking gel buffer	5.0 ml	-
1.5% Ammonium per sulfate	1.0 ml	1.5 ml
10% SDS	0.2 ml	0.3 ml
Water	11.3 ml	14.45 ml
TEMED	0.015 ml	0.015 ml

subjected to side strip staining by Copper chloride as described earlier (Sections 5.2.6.2 and 5.2.6.3). The resolving gel is cut horizontally into two parts perpendicular to the direction of the run at appropriate distance. The lower part containing low molecular weight proteins is separated from the upper part containing high molecular weight proteins. Each part of the gel is macerated separately and loaded on to the specially designed tube holding a column of polyacrylamide gel of appropriate concentration. Low molecular weight proteins are loaded on 10% polyacrylamide gels, while high molecular weight proteins are loaded on 5% polyacrylamide gels and electrophoresed. The separating proteins are eluted out in 0.125M Tris-HCl buffer pH 6.8 containing 0.1% SDS.

6.2.2. On-Line Elution of Proteins During Electrophoresis

Using the apparatus described in the previous chapter (Section 5.2), the sample is electrophoresed through the column of

polyacrylamide gel at 20mA. Once the tracking dye reached the gap just above the gel plug, the elution is started at a flow rate of 1ml/min. When low molecular weight proteins are being separated, 140 fractions of 2 ml each are collected, whereas, for the high molecular weight proteins, the number of fractions collected is 275. The optimal density of the fractions collected is recorded on a Beckman DU-6 spectrophotometer at 280nm.

6.2.2.1 Composition of reagents used in casting the gels for electroelution

Reagents (ml)	Stacking	Resolving 5%	Gel plug 10%	
Acrylamide-Bisacrylamide (30:0.8)	2.5	5.0	10.0	6.9
3.0M Tris - HCl (pH8.8)	-	3.75	3.75	-
0.5M Tris - HCl (pH6.8)	5.0	-	-	2.5
1.5% Ammonium per sulfate	1.0	1.5	1.5	0.5
10% Sodium dodecyl sulfate	0.2	0.3	0.3	0.1
Water	11.3	19.45	14.45	-
TEMED	0.015	0.015	0.015	0.015

The homogeneity of the fractions obtained are analyzed by SDS-PAGE. Selective fractions of interest are subjected to peptide mapping to check for sequence homology.

6.2.3. Peptide Mapping

This is a very accurate technique to establish if two proteins have segments of common amino acid residues. The basic principle behind this technique is the cleavage of the parent protein by

specific physical, chemical or biochemical methods and separating the peptides on SDS-PAGE. Comparison of the proteins obtained for two parent protein yields information on the sequence homology. One of the most important advantages of using this technique is the small amount of sample required for the purpose.

Of the chemical methods, cyanogen bromide (cleaves methionine- x bonds), hydroxylamine (cleaves asparagine - glycine bonds), N-bromosuccinimide (cleaves tryptophan-x bonds), partial acid hydrolysis (cleaves x asparagine - proline bonds), and partial basic hydrolysis (cleaves serine-x bonds) are in use.

Physical method involves heating the purified protein at 110°C at pH 6.8 for 1-2 hours; this cleaves the asparagine-proline bonds.

The biochemical cleavages use proteinases effectively to cleave specific peptide bonds. Staphylococcus aureus V8 proteinase at pH4 to 8 cleaves the glutamine - x and asparagine - x bonds; α-chymotrypsin cleaves tryptophan, tyrosine, phenylalanine and leucine bonded to any other amino acid at an optimal pH range of 7 to 9; trypsin acts on arginine and lysine connected to other amino acids at an optimal pH of 8 to 9; subtilisin and pronase are broad specificity proteinases, while Papain acts on bonds between lysine, arginine, leucine, glycine and phenylalanine and other amino acids with the optimal pH range of 7 to 8.

In the current study, two methods, one chemical and one biochemical, have been used to study relationships between proteins purified from epididymal sperm membranes.

6.2.3.1 Peptide mapping using chymotrypsin (Andrews, 1990)

The procedure exploits the enzymatic property of chymotrypsin in cleaving peptide bonds between tryptophan, tyrosine, phenylalanine, leucine and other amino acids.

Reagents

Tris-HCl Buffer 0.125M (pH 6.8) containing 0.5% SDS, 10% glycerol and 0.001% bromophenol blue.

Chymotrypsin 1mg/ml in 0.125 M Tris-HCl (pH 6.8) containing 0.5% SDS, 10% glycerol and 0.001% bromophenol blue.

Procedure

An aliquot of the homogeneous fraction of protein (containing 10μg protein) is diluted in 0.125M Tris-HCl buffer (pH 6.8) containing 0.1% SDS is taken and dried in a Heto Vac. The sample is reconstituted with 0.125M Tris-HCl buffer (pH 6.8) containing 2% SDS and boiled for 3 min.

To the boiled samples in a final volume of 40μl, 10μl of chymotrypsin (1mg/ml) is added and the mixture is incubated at 37°C for 30 minutes. Thereafter 2-mercaptoethanol (to a final concentration of 10%) and SDS (to a final concentration of 2%) are added to the samples and boiled for 2 minutes to terminate proteolysis.

The fragmented peptides so obtained are run on SDS-PAGE (15% gels) using Laemmli's buffer system. The gels are stained by Coomassie brilliant blue R-250 (0.2%) in 40% methanol and 10% acetic acid.

After one hour, destaining is done by diffusion in 10% Acetic acid and 5% methanol.

Composition of reagents for the preparation of 15% SDS-PAGE gel:

Reagents	Stacking gel	Resolving gel (15%)
Acrylamide-Bisacrylamide (30:0.8)	2.5 ml	15 ml
Resolving gel buffer	–	3.75 ml
Stacking gel buffer	5.0 ml	–
1.5% Ammonium persulfate	1.0 ml	1.5 ml
10% SDS	0.2 ml	0.3 ml
Water	11.3 ml	9.45 ml
TEMED	0.015 ml	0.015 ml

6.2.3.2 Peptide mapping by Cyanogen Bromide (Huang et al., 1983)

Tryptophan residues because of their relatively low abundance, provide ideal sites for specific chemical cleavage of proteins. The ability to split proteins into only a few large fragments not only simplified isolation of the peptides for subsequent amino acid analysis, but also simplifies the task of peptide alignment.

The mechanism of action is based on the fact that the reduced and alkylated protein or peptide is treated with dimethyl sulfoxide in hydrochloric acid (DMSO/HCl) to oxidise tryptophan to oxindolylalanine and methionine to the corresponding sulfoxide. This reaction is partially neutralized, and cyanogen bromide (CNBr) in acetic acid is added to cleave on the carboxyl side of oxindolylalanine.

This method for cleavage at tryptophan residues is close to quantitative yields and the yield is apparently unaffected by the structure of either the amino or carboxyl residues adjacent to the tryptophan being cleaved. The reagents used are volatile and stable and the reaction time is short as compared to that for other tryptophan cleavages.

Reagents

Oxidation solution: 75ml glacial acetic acid, 37.5ml 9M hydrochloric acid and 10ml dimethylsulfoxide mixed fresh before use.

15M Ammonium hydroxide chilled at $-20^{\circ}C$

5M Acetic acid

Cyanogen bromide: 0.3g/ml of cyanogen bromide in 5M acetic acid.

Procedure

The protein sample suspended in 0.125M Tris-HCl buffer (pH 6.8) containing 0.1% SDS is brought to pH1.5 with HCl and lyophilized.

To $2\mu g$ of the lyophilized protein sample, 4.9 ml of the oxidation mixture is added and incubated for 2 hours at $4^{\circ}C$.

The sample is chilled in an ice bath and 4.4ml of 15M ammonium hydroxide chilled to $-20^{\circ}C$ is added very carefully.

Thereafter, 40ml of CNBr solution is added. The flask is then capped tightly and sealed with parafilm. The reaction is allowed to proceed for 30 hours at $4^{\circ}C$ in dark. The fragmented peptides are precipitated using methanol and subjected to SDS-PAGE on 15%

gels. Comparison of the patterns of the fragmented peptides in different samples of purified homogeneous proteins helps in establishing the extent of sequence homology.

6.2.4 Immunological Studies (Harlow and Lane, 1988)

Polyspecific antibodies are raised against a 31kD protein isolated from the membrane of cauda epididymal sperm. The cross reactivity of this antibody with high molecular weight proteins isolated from membranes of caput sperm is checked using the double immunodiffusion technique.

6.2.4.1 Production of Antibodies

6.2.4.1.1 Animals: Antibodies are generated in four to six month old white female rabbits of New Zealand breed purchased from the Indian Drugs and Pharmaceuticals Limited, Rishikesh. They are allowed to get acclimatized to the lab conditions over a sufficient period of time before being immunized.

6.2.4.1.2 Collection of preimmune sera: The rabbit is restrained by placing it on a large towel and wrapping it around the rabbit such that it can be held conveniently for bleeding. The marginal vein is located and massaged vigorously so that it stands out prominently. A patch of hair about 2/3 of the distance from the head to the tip of the ear is shaved using a clean razor blade.

A gentle nick is made at an angle of 45° to the vein. Care is taken that the cut is not too deep. The blood oozing out is collected in a clean glass test tube with a wide mouth (1.5 cm diameter). Any clot formed during collection of blood is wiped

clean with cotton swabbed in clean warm water. Once enough blood is collected, the bleeding is stopped by gentle pressure to the cut using a sterlized piece of cotton wool.

6.2.4.1.3 Serum preparation: The blood collected by the above method is allowed to clot for 30 - 60 minutes at $37^{\circ}C$. The clot is detached from the sides of the test tube by rimming it gently with a pasteur pipette. The clot is then allowed to contract at $4^{\circ}C$ overnight. The serum is separated from the clot and any other insoluble material by centrifugation at 10,000 xg for 10 minutes at $4^{\circ}C$.

6.2.4.1.4 Sample preparation for immunization

Pretreatment of the dialysis tubing

1. The dialysis tubing from Sigma with a cut off of 3000Mr was cut at an appropriate length.

2. The tubing was then placed in 500ml of 0.2M sodium bicarbonate containing 0.005M ethylene diamine tetraacetic acid.

3. The tubing was boiled for 5 min and the solution poured out thereafter. The drained solution was replaced with a fresh solution of sodium bicarbonate and ethylenediamine tetracetic acid and and the tubing boiled again for 5 min.

4. The second wash was also discarded and the tubing washed extensively in distilled water and the washed tubing was placed in large volume of deionized water and covered.

5. The tubing was then autoclaved for 10 minutes on a liquid cycle and stored at 4°C. After cooling 0.02% sodium azide was added to prevent microbial growth.

Dialysis

The fraction number 75 containing the 31kD protein is dialyzed extensively (8 hours) against water to remove the Tris-HCl buffer and 0.1% SDS present in the sample. Protein concentration is estimated in the dialysed sample and the sample is lyophilized to dryness. The samples are resuspended in 0.04M phosphate buffer saline pH 7.0.

6.2.4.1.5 Immunization

A suitable aliquot (~0.5 ml) containing 100μg of protein of the reconstituted protein in phosphate buffered saline is put in an eppendorf tube (1.5 ml capacity whose cap is pricked with tiny holes for the passage of the syringe needle). To this another 0.5ml of Complete Freund's adjuvant is added, and the mixture is initially mixed with gentle vortexing. However, to generate a good emulsion of the adjuvant's oil in the phosphate buffered saline containing the sample, a prolonged mixing is needed. This is done by drawing the oil – water mixture into a syringe and ejecting it forcefully back into the eppendorf tube. The emulsion is ready when it forms a tight non dispersing drop when placed on the surface of saline.

The rabbit to be immunized is immobilized with a large towel wrapped around it such that its back is exposed. Tiny patches of

fur at five sites along the back of the rabbit are shaved. The skin of the rabbit is pinched between the thumb and the forefinger and gently lifted. The needle is inserted into the space created by this lifting and 0.2 ml of the emulsion is injected at the site by depressing the plunger. The needle is withdrawn and the site of injection is gently rubbed between the forefinger and the thumb to stop any inoculum from escaping. Similar subcutaneous injections are administered at the other four sites.

6.2.4.1.6 Sampling serum: Seven days after the first set of injections at multiple sites, a sample bleeding is done to check for the production of antibody. Only the rabbits that showed good immune response are administered the booster and finally bled to obtain polyspecific antiserum.

6.2.4.1.7 Booster doses: 50 μg of purified protein is injected as booster at 7 and 14 days after the first set of injection. The booster doses use Incomplete Freund's adjuvant in contrast to the Complete Freund's adjuvant used for the initial injection. Final bleeding is done seven days after the second booster dose and the serum containing polyspecific antibodies isolated for further investigation.

6.2.4.2 Double immunodiffusion

This is a method originally developed by Ouchterlony (1962). Briefly, the method involves diffusion of antigen and antibody in an agarose gel. The formation of antigen-antibody complexes are detected as precipitin lines within the gels. At times the

precipitin lines are so faint that for photographic purposes staining becomes imperative.

Reagents

(a) Agarose 2% (w/v) in water: 2g agarose is weighed and dissolved by warming up in 100 ml distilled water.

(b) Immunodiffusion buffer (2x): 0.3M sodium chloride containing 40mM sodium phosphate buffer pH 7.0 and 0.2% sodium azide to prevent microbial growth

(c) 0.15M sodium chloride

(d) Staining solution: 0.05% Coomassie brilliant blue R250 in 40: 10:50 mixture of methanol, acetic acid and water respectively.

(e) Destaining solution: 5 : 10 : 85 mixture of methanol, acetic acid and water.

Procedure

Equal volumes of 2% agarose and diffusion buffer are transferred to a 25 ml conical flask. The two solutions are mixed at 50°C and poured between two glass plates (10 x 10cm) separated by spacers of 1.5mm thickness. After complete polymerization of the gel at room temperature, the spacers and one of the glass plates are removed. One central and four peripheral wells are punched equidistant from each other. The antibody is loaded in the central well and the cross reacting antigen to be tested are dispensed into the peripheral wells. The gels are incubated in a humidifying chamber at 37°C overnight by which time the cross reacting antigen-antibody complexes show precipitin lines.

6.2.4.2.1 Staining gels for proteins

Precipitin lines in agarose gel can easily be identified against a dark background. The resolution can however be enhanced by staining. For this purpose, the unreacted proteins present in the agarose gel first must be removed. This is achieved by the following method.

The sample wells of the gel to be stained are filled with a drop of saline. The plate containing the gel is placed on a flat surface. Over this a piece of whatman 3mm paper (10 x 10cm) is placed carefully without trapping any air. On this, a wad of adsorbant paper of the same dimension is placed, and this is covered by a glass plate. A large reagent bottle approximately 1 kg in weight is placed on this glass plate. Ten minutes later, all the layers are very carefully peeled off the gel surface to ensure that the gel remains undamaged. The pressed gel is immersed in saline for 20 minutes. The saline is replaced by a fresh supply for another 20 minutes, which is then replaced by distilled water for 20 minutes. The gel is removed to a glass plate and dried in an incubator. The dried gel is stained with the Coomassie brilliant blue stain for 20 minutes and destained thereafter by adding the destaining solution.

6.3 RESULTS

Sperm membrane proteins from the three segments of the epididymis have been solubilized in Laemmli's buffer (1970) containing 2% SDS, 5% β- mercaptoethanol, 20% glycerol in Tris-HCl buffer (pH 6.8) at 100°C for 3 min. The solubilized proteins have been run

on a 10% preparative gel. Appropriate sections of the gel containing high and low molecular weight proteins were fractionated on 5% and 10% SDS-PAGE columns respectively in the specially designed apparatus discussed in the previous chapter.

Fig. 6.1(a,b,c) shows the fractionation of low molecular weight proteins from caput, corpus and cauda respectively. A comparison of the fractions obtained in each case brings forth some interesting features. Proteins eluting in fractions number 10, 14, 20, 25, 29, 39, 52 and 112 are present in the elution profiles of all the three segments. The protein eluting out in fraction number 38 and 43 is present in the elution profiles of caput and corpus, but is absent from the cauda sperm membrane low molecular weight protein eluates. Similarly, proteins eluting in fractions number 71, 92, and 107 are present only in the profile of the caput sperm eluate, and are conspicuous by their absense from the elution profiles of corpus and caput sperm. 34 and 101 fraction proteins are present only in corpus elution profile, while the 83 and 103 fraction proteins are specific to cauda profile. A host of proteins eluting in fractions obtained from the low molecular weight elution profiles of corpus and cauda is apparent. These include the 61, 63, 69, 75, 80-83, 98, 106 and 108 fraction proteins.

The elution profiles of fractions obtained from the elution of high molecular weight proteins of caput, corpus and cauda fractionated on a 5% SDS-PAGE column are give in Fig. 6.2(a,b,c). As in the case of low molecular weight proteins, the high

molecular weight components also exhibit such trends as the appearance of new fractions, continued presence of certain fractions and the loss of certain fractions. Three proteins eluting in fraction numbers 113, 122 and 201 are absent from the elution profile of high molecular weight proteins of the caput epididymis but are present in the corpus and cauda eluates.

Proteins eluting in band numbers 213, 209, 206, 188, 156, 153, 147, 141, 132, 128, 117, 100 and 92 are present in the elution profiles of all the three segments. In contrast, fraction numbers 107, 110, 170, 192, 195, 216, 218, 222, 230 and 236 present in the proximal segments of epididymis, elution profiles are notable by their absence from the elution profile of the cauda epididymis. Chymotrypsin digestion of selected proteins is given in Figs. 6.3 and 6.4. From these Figures, it is apparent that there is no sequence homology between the polypeptide fragments of the fraction numbers 34, 83, 61, 98 and 103 (of the low molecular weight fractions from cauda epididymal sperm) and fraction number 125, 192 and 226 (of the high molecular weight protein fractions from caput).

However, from Fig. 6.4, it is evident that there is a sequence homology between fraction number 75 (of low molecular weigh cauda proteins) and 170 (high molecular weight caput proteins). The fraction number 170 protein is cleaved into 9 fragments while the fraction number 75 protein is cleaved into a fragments. All the 9 fragments move with the relative mobility of 9 corresponding fragments in the fraction number 170. This is indicative of the

possibilty that the fraction 75 protein is a part of the fraction 170 protein in terms of sequence homology.

The cyanogen bromide digest of the 75 and 170 fraction proteins convincingly proves that there is sequence homology between the two proteins (Fig. 6.5). Fraction numbers 75 and 170 after on CNBr-DMSO/HCl treatment give 4 and 7 fragments respectively. The Rf values of 3 out of 4 fragments of fraction 75 find corresponding 3 fragments out of 7 from fraction 170 moving with similar Rf values.

From the molecular weight determination it is concluded that the fraction 170 high molecular weight protein corresponds to 124 kD while the low molecular weight fraction 75 corresponds to a molecular weight of 31 kD (Fig. 6.6a,b).

Polyspecific antibodies raised against the 31 kD protein gives a precipitin band with the 124 kD protein thereby, establishing that the two share common antigenic determinants. The cross reactivity of the polyspecific antibody against the 31kD protein with other high molecular weight protein fractions from caput is nil (Fig. 6.7a,b,c).

6.4 Discussion

The elution profiles show convincingly that the apparatus designed for the on-line elution of proteins during electrophoresis can be very useful for the purification and analysis of sperm membrane proteins. Not only is the separation good, but the elution profiles show that the results are highly reproducible. The

Fig. 6.1   The Elution Profile of Low Molecular Weight Sperm
Membrane   Proteins   separated   by   the   on-line
electroelution on 10% SDS-PAGE column electrophoresed at
20 mA constant current and eluted at a flow rate of 1
ml/min.

(a) Low molecular weight proteins of caput epididymal
sperm membrane.

(b) Low molecular weight proteins of corpus epididymal
sperm membrane.

(c) Low molecular weight proteins of cauda epididymal
sperm membrane.
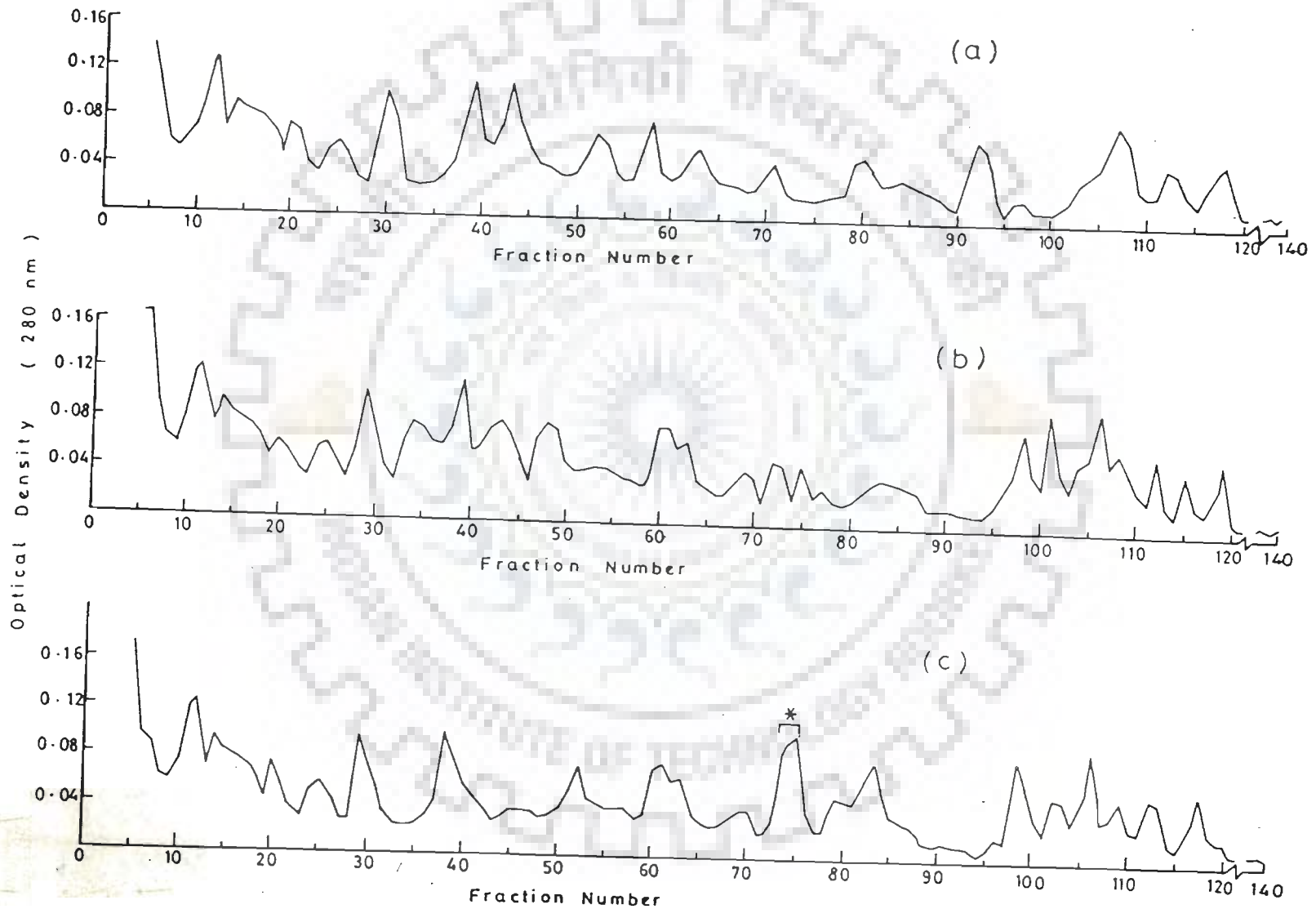
FIG. 6·1

Fig. 6.2   The Elution Profile of High Molecular Weight Sperm Membrane Proteins separated by electroelution on 5% SDS-PAGE column at a constant current of 20 mA and flow rate of 1 ml/min.

(a)   High molecular weight proteins of caput epididymal sperm membrane.

(b)   High molecular weight proteins of corpus epididymal sperm membrane.

(c)   High molecular weight proteins of cauda epididymal sperm membrane.
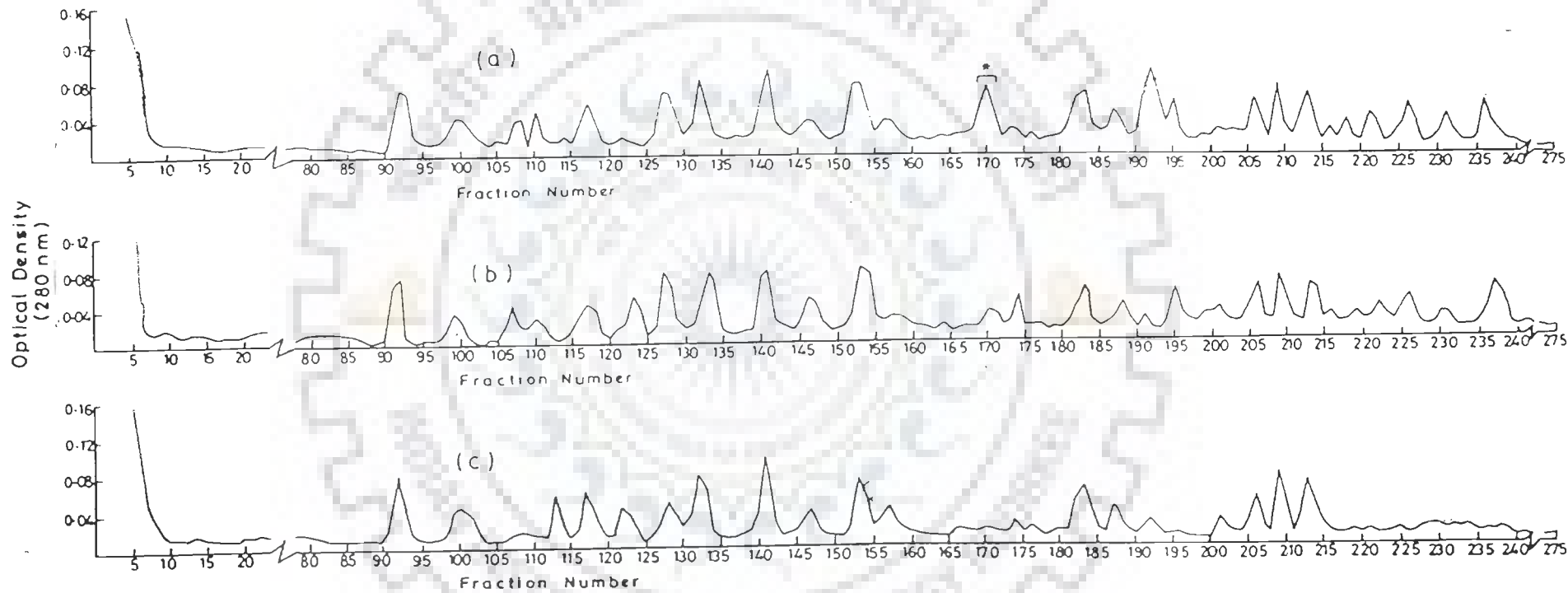
FIG 16.2

Fig. 6.3   Peptide Maps of Sperm Membrane Proteins Following
Chymotrypsin Digestion. 10 µg protein from Fraction
Number 34, 83, 61, 98 and 103 of low molecular weight
protein eluates of cauda epididymis and fraction number
125, 192 and 226 of the high molecular weight protein
eluates are subjected to chymotrypsin digestion using 10
µg chymotrypsin, at 37°C for 30 min.   The Protein
fragments so obtained are separated on 15% SDS-PAGE.

Fig. 6.4   Chymotrypsin Digestion of Fraction Number 75 and 170
Peptide maps of Fraction Number 75 from the low
molecular weight proteins eluates of cauda epididymis
and Fraction number 170 from the high molecular weight
proteins of caput epididymis obtained after chymotrypsin
digestion at 37°C for 30 mins resolved on 15% SDS-PAGE.

Fig. 6.5   Cyanogen Bromide Peptide Mapping. The Fraction number
75 of low molecular weight protein eluates of cauda
epididymis and Fraction number 170 of high molecular
weight protein eluates of caput epididymis are subjected
to peptide mapping by CNBr-DMSO/HCl and the fragments
obtained are separated on 15% SDS-PAGE.
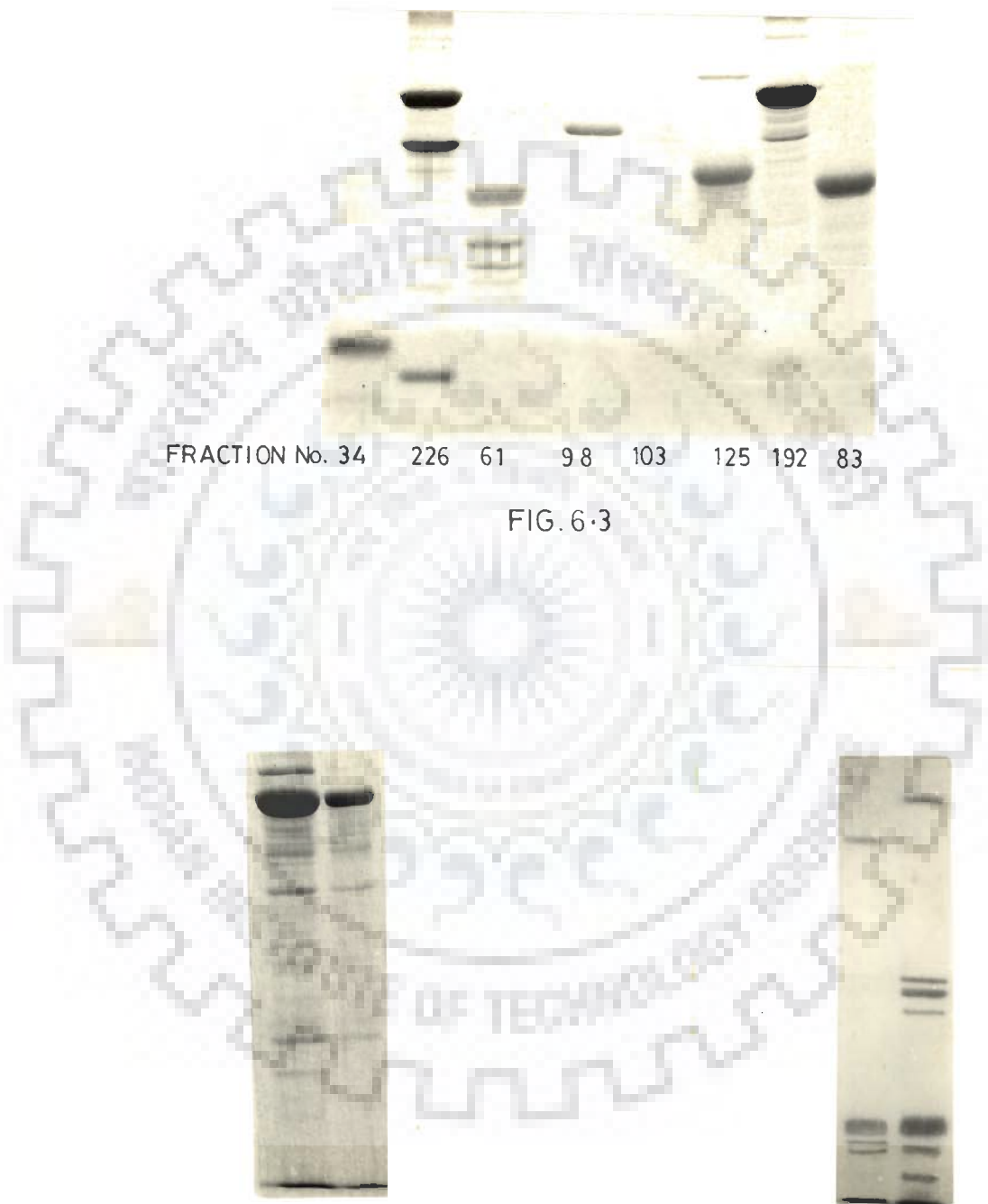
FRACTION No. 34   226 61   98   103   125 192 83

FIG. 6·3

FRACTION No.   170   75

FIG. 6·4

75   170

FIG. 6·5

Fig. 6.6   The   Molecular   Weight   Determination   of   Fractions
            Exhibiting Sequence Homology

    (a)   The Fraction number 75 of low molecular weight
           cauda protein eluates (Lane 1) and Fraction number
           170 of high molecular weight protein eluates of
           caput epididymis (Lane 2) and molecular weight
           markers (Lane 3) are run on 10% SDS-PAGE.

    (b)   Calibration curve of polypeptide Molecular weight
           versus Relative Mobility on 10% SDS-PAGE.   The
           molecular weight markers used are Lysozyme (14.4
           kD), Carbonic Anhydrase (29 kD), Ovalbumin (45 kD),
           Bovine Serum Albumin (66 kD) and Phosphorylase b
           (97.4 kD).   The Rf of 0.19 and 0.68 correspond to
           molecular weights of 124 kD and 31 kD respectively.

(a)
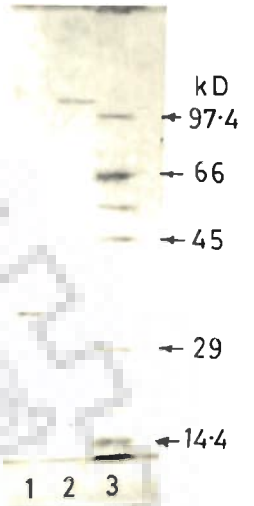
(b)
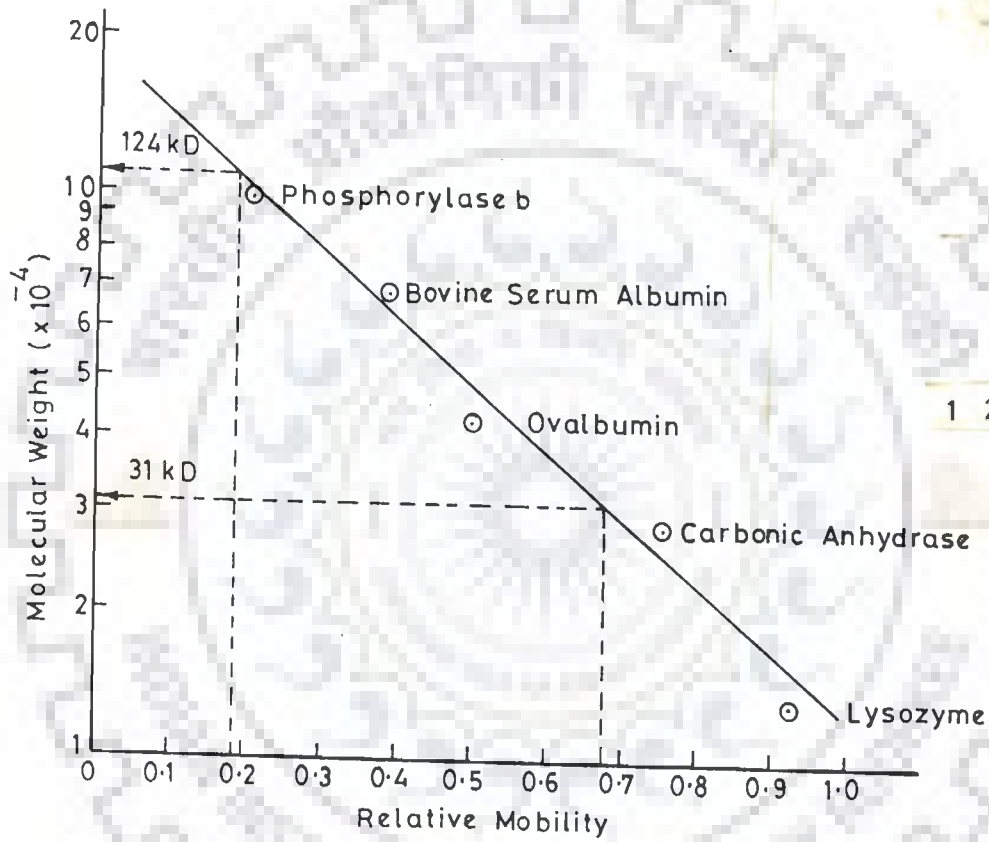
FIG. 6·6

Fig. 6.7   (a) Double Immunodiffusion Patterns of Purified 31kD
                 Protein From Cauda Epididymal Sperm Membrane with
                 Anti 31-kD Polyspecific Antibody.   The central well
                 of 1% agarose plate contains 20 μl of Anti-31kD
                 protein.   The peripheral wells contain 20 μl each
                 of (a) 31 kD protein, (b) 124 kD protein, (c)
                 Preimmune sera and (d) PBS.   The gel has been
                 stained with Coomassie brilliant blue R-250.

            (b) Double Immunodiffusion Patterns of Anti 31 kD
                 Protein (central well) with 20 μl each of fraction
                 numbers 107 (well a), 110 (well b), 192 (well c) of
                 high molecular weight protein eluates from caput
                 epididymis and PBS (well d).   The gel has been
                 stained with Coomassie brilliant blue R-250.

            (c) Double Immunodiffusion Patterns of 20 ml Anti-31 kD
                 Protein (central well) with 20 μl each of Fraction
                 Number 195 (well a), 216 (well b), 218 (well c) of
                 high molecular weight protein eluates from caput
                 epididymis and PBS (well d).   The gel has been
                 stained with Coomassie brilliant blue R-250.

            (d) Double Immunodiffusion Patterns of 20 ml Anti-31 kD
                 Protein (central well) with 20 μl each Fraction
                 Number 222 (well a), 230 (well b), 236 (well c) of
                 high molecular weight protein eluates from caput
                 epididymis and PBS (well d) stained with Coomassie
                 brilliant blue R-250.
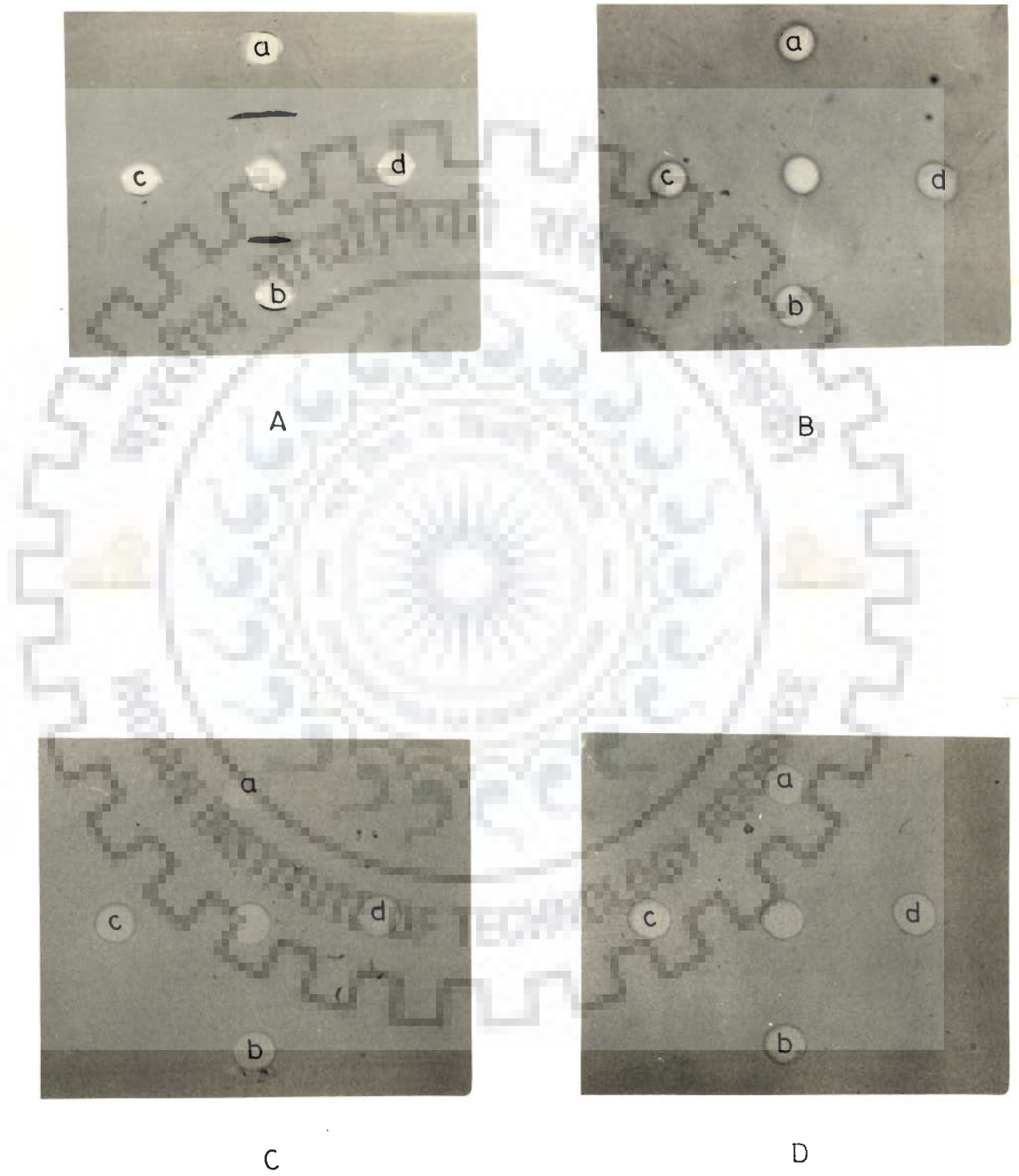
FIG. 6-7

elution patterns obtained for membrane proteins isolated from the caput, corpus and cauda sperm provide an excellent means of comparing and identifying the precise differences in the protein profile. An additional advantage is that the procedure is non destructive and the recovery of specific proteins from the complex mixture of sperm membrane protein is greatly simplified.

The results have amply demonstrated that a 10% polyacrylamide column for low molecular weight proteins and a 5% polyacrylamide column for high molecular weight proteins are ideal for good resolution. The elution profiles reveal muchinformation about the changing proteins of sperm membranes in the epididymis.

Most proteins were found to be common to sperm membranes irrespective of the location in the epididymis from where sperm was collected. However, the profiles of membrane proteins of caput, corpus and cauda sperm are not entirely identical. The differences in the protein profiles could be because certainproteins arelost from the sperm membrane,while others are added during passage through the epididymis. The absorptive (Moore and Bedford, 1979; Goyal and Hrudka, 1980; Dacheux and Voglmayr, 1983; Hermo and Morales, 1984; Turner, 1984; Fain-Maurel et al., 1988 and Veeramachaneni et al., 1990) and secretory (Koskimies and Kormano, 1975; Cameo and Blaquier, 1976; Flickinger, 1983 and Fournier-Delpech et al., 1988) nature of the epididymal epithelium facilitates this process. The proteins shed from the sperm membranes could either be absorbed directly as such, or could be acted upon by enzymes in the epididymal lumen

and the degraded fragments taken up by the epididymal epithelium. Similar suggestions based on experimental evidence have been provided in the past (Attramadal et al., 1981; Djakiew et al., 1985; Robaire and Hermo, 1988 and Jones, 1989b). It appears that the proteins gained by the sperm membranes are of epididymal origin since proteins not present in caput sperm membranes are detected in caudal sperm membranes. On earlier occasions too, the epididymis has been shown to synthesize and secrete proteins into its lumen for action or incorporation into sperm (Moore, 1980; Brooks, 1981b, 82, 85; Voglmayr et al., 1980, 83, 85 and Dacheux et al., 1989). Thus, the epididymis seems to be fully equipped for transforming the sperm surface protein are.

The most significant observations of the detailed analysis of sperm membrane protein is the valuable information obtained on the modification of pre-existing sperm membrane components. When a careful examination of the membrane proteins of caput, corpus and cauda sperm is made, differences are observed with respect to 4 proteins in the high molecular weight range and 6 proteins in the low molecular weight range. Proteolytic cleavage of each of these ten proteins by both enzymatic and chemical methods, followed by subsequent mapping of the resultant peptide fragments on SDS-PAGE, revealed the extent of sequence homology. Some fragments of the 31 kD protein from cauda sperm membrane had identical electrophoretic mobility as fragments of a 124 kD protein from caput sperm membranes. This suggest that the two proteins are somehow related.

It appears that these proteins are firmly anchored in sperm membranes since both the 31 kD and 124 kD proteins are solubilized by SDS. Further, antibodies raised to the 31kD protein cross reacted with only the 124 kD protein, implying that these two proteins share some common epitopes. At this stage it would be interesting to recall that proteolytic enzymes have in the past been held responsible for biologically activating proteins by cleavage (Danzo, 1986; Hendry III and Danzo, 1986; Hardy et al., 1987 and Topfer-Petersen et al., 1990a,d). In the epididymis too the presence of proteolytic enzymes has been demonstrated (Danzo, 1986; Hendry III and Danzo, 1986 and Lessley and Garner, 1990) and unmasking of sperm surface components by proteolytic cleavage is believed to be a process in epididymal sperm maturation ((Blobel et al., 1990; Okamura et al., 1992 and Nehme et al., 1993). It is possible that the 124 kD protein of the caput sperm membrane is acted upon by proteolytic enzymes present in the epididymal lumen to generate a 31 kD protein still anchored in the membrane. Obviously, such a mechanism takes place as the sperm descend from the caput to the cauda epididymis. Such a processing of existing sperm membrane proteins by proteolytic cleavage perhaps permit better interaction between the sperm and the ova.

6.5 CONCLUDING REMARKS

1. Membrane protein of sperm collected from the caput, corpus and caudal segments of the goat epididymis have been analysed.

2. Proteins were separated on polyacrylamide gels and eluted into buffer using a self-designed protein purification assembly.

3. A 31 kD protein was identified which was unique to membranes of only the sperm collected from the cauda epididymis.

4. Polyspecific antibodies raised to this 31kD protein reacted with a 124 kD protein isolated from the membranes of caput sperm.

5. Peptide mapping of the 31kD and 124 kD proteins by chymotrypsin digested and CNBr coupled DMSO/HCl cleavage showed fragments indicative of sequence homology in these two proteins.

6. It is suggested that the 124 kD sperm membrane protein is transformed into the 31 kD protein by proteolysis in the epididymis and could be associated with sperm maturation.

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