

**ISOLATION AND CHARACTERIZATION OF THE
SACCULAR ELEMENTS OF CYTOPLASMIC DROPLETS
FROM RAT EPIDIDYMAL SPERMATOOA.**

by

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*To Sprague-Dawley rats
and all other organisms used
for scientific research*

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ABSTRACT

The cytoplasmic droplet, a small localized outpouching of cytoplasm of epididymal spermatozoa of unknown biological significance, contains numerous saccular elements as the near exclusive membranous component. These saccules were isolated by subcellular fractionation to raise antibodies which immunolabeled not only saccules of the droplet in situ but also Golgi apparatus in somatic epithelial cells lining the epididymis. Immunocytochemical studies at the light and electron microscopic levels further revealed the saccular elements as the exclusive site of reactivity for Golgi apparatus markers IGN 38, α 2,6 sialyltransferase and β 1,4 galactosyltransferase. The isolated droplet fraction was enriched in galactosyltransferase and sialyltransferase activities and endogenous glycosylation assays identified the modification of several endogenous glycopeptides. In situ lectin binding assay on electron microscopy demonstrated D-galactose and N-acetyl galactosamine constituents in the saccules within the droplet and also in the adjacent plasma membrane. The glycosylating activities in the saccular elements may be related to plasma membrane modifications which occur during epididymal spermatozoa maturation.

RÉSUMÉ

La gouttelette cytoplasmique est une petite masse de cytoplasme présente le long de la queue des spermatozoïdes épидидymaires. Cette gouttelette contient presque exclusivement des saccules aplatis dont la fonction n'est pas encore précisée. Nous avons procédé à leur isolation par fractionnement cellulaire et préparé des anticorps à partir de ces fractions. Par immunocytochimie nous avons observé que ces anticorps réagissent non seulement avec les saccules des gouttelettes mais également avec l'appareil de Golgi des cellules épithéliales du canal épидидymaire. De plus des anticorps anti-TGN-38, anti- α 2,6 sialyltransférase et β 1,4 galactosyltransférase, des marqueurs de l'appareil de Golgi, réagissent également avec les saccules des gouttelettes. Des gouttelettes isolées montrent une activité de la galactosyltransférase et de la sialyltransférase et une modification des glycopeptides endogènes par glycosylation. L'utilisation de lectines nous ont finalement permis de démontrer, par cytochimie en microscopie électronique, que le D-galactose et l' N-acetyl galactosamine sont des composants des saccules des gouttelettes et de la membrane cytoplasmique adjacente. La glycosylation observée dans les éléments sacculaires doit être associée aux modifications biochimiques de la membrane cytoplasmique des spermatozoïdes qui prennent place au cours de leur maturation le long du canal épидидymaire.

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ABBREVIATIONS

CD	Cytoplasmic droplet
EM	Electron microscopy
ER	Endoplasmic reticulum
GPI	Glucose phosphate isomerase
GT	Galactosyltransferase
HK	Hexokinase
kD	Kilodalton
LDH	Lactate dehydrogenase
LM	Light microscopy
SE	Saccar elements of cytopalsmic droplet
ST	Sialyltransferase
TGN	Trans Golgi Network

INTRODUCTION

INTRODUCTION

At spermiation, spermatozoa within testis are non-motile and infertile. It is only after their transit through the epididymal tract that motility and fertility are acquired. One of the most conspicuous morphological changes to occur to spermatozoa during their transit through the epididymis is the displacement of the cytoplasmic droplet along their tails (Fig 1), and its subsequent detachment into the lumen of the corpus and cauda epididymidis (Hermo et al, 1988). The cytoplasmic droplet is a small mass of cytoplasm that is retained by spermatozoa as they are released from the seminiferous epithelium of the testis during spermiation. Cytoplasmic droplets are ubiquitous to all mammalian species (Branton and Salisbury, 1947, Nicander 1957, Hafez and Prasad, 1976) and also to some non-mammalian ones (Bedford, 1979).

Infertility is very often found to be caused by various genetic and environmental-induced defects of the tail. Barth and Oko (1989) reviewed that many of these defects are associated with the trapping of the cytoplasmic droplets in the abnormal bend of the sperm's tail. Studies have shown that ejaculates containing a high proportion of spermatozoa with attached droplets are correlated with altered epididymal function and reduced fertility (reviewed by Hermo et al, 1988), further suggesting a connection between the function of cytoplasmic droplet and fertility.

The bulk of the late spermatid's cytoplasm, referred to as the residual body, which is not retained by spermatozoa but detached from it during spermiation, is phagocytosed and disintegrated rapidly by the Sertoli cells lining the seminiferous epithelium (Morales et al, 1985). The segregation of the cytoplasmic droplet and residual body appears to be specific, with the flattened membranous elements, termed saccular elements, being consistently retained in the cytoplasmic droplet but not found in the residual body, while organelles such as mitochondria, ribosomes, remnants of the endoplasmic reticulum and lipid bodies are segregated into the residual body. This phenomenon not only suggests that phagocytosis of the residual body by the Sertoli cell involves a specific and selective mechanism, but it also leads logically to the speculation that the saccular elements selectively retained in the

cytoplasmic droplet by spermatozoa may play a biological role

Since its original discovery by Retzius (1909), efforts have been devoted to investigate different aspects of the cytoplasmic droplet. Ultrastructural studies of the cytoplasmic droplets revealed that numerous short, straight or C-shaped, flattened saccular elements were its sole content. Although speculations on the identity of these membranous elements as disintegrating Golgi elements (Weigl, 1912, Gatenby and Woodger, 1921, Bell, 1929, Gatenby and Collery, 1943, Gresson and Zlotnik, 1945 & 1948, Cavazoa and Melampy, 1954, Bloom and Nicander, 1961) or endoplasmic reticulum (Bloom and Nicander, 1961) have been made, these elements have neither been isolated nor biochemically characterized. However, biochemical studies revealed the presence of various enzyme activities in isolated cytoplasmic droplets. In particular, lysosomal enzyme activities were first found by Dott and Dingle (1968) and later confirmed by Garbers et al (1970) and Kaplan et al (1984). This has led to the view that the cytoplasmic droplet is a lysosomal derived organelle and that the saccular elements within may be lysosomes. The fate of the droplets after being shed from spermatozoa in the distal portion of the epididymis was also investigated. It appeared that the contents of detached droplets were taken up by the epithelial clear cells lining the epididymal epithelium (Temple-Smith, 1984, Hermo et al, 1988).

Despite various investigations performed on the droplet, the two fundamental questions regarding the cytoplasmic droplets still remain unanswered. First, what is the true identity of the saccular elements found within the cytoplasmic droplets? The ubiquity of cytoplasmic droplets in various mammalian species and its peculiar displacement along the sperm tail, before its subsequent detachment during spermatozoa epididymal transit, raise the second question: does the cytoplasmic droplet have a biological function in the maturation of spermatozoa? As the saccular elements are the near exclusive membranous components of the cytoplasmic droplet, it is presumed that the biochemical characterization of this organelle may provide an insight into the function of the cytoplasmic droplet.

There are several objectives of the present study. First, to determine to what structures the saccular elements within the cytoplasmic droplets correspond, the

cytoplasm of spermatids at late steps of spermiogenesis will be analyzed at the electron microscopic level. The second objective of this study is to evaluate the hypothesis that the saccular elements are Golgi elements. For this purpose, we first sought to isolate, by subcellular fractionation, the saccular elements of the cytoplasmic droplet, to raise antibodies against these components and to analyze their polypeptide composition with the combined use of polyacrylamide gel electrophoresis and immunocytochemical methods applied to Western blots. In addition, we demonstrate, by morphological analysis on light and electron microscopy combined with immunocytochemical studies, that the cytoplasmic droplet contains loosely organized saccules retaining Golgi characteristics. The final objective of this study is to evaluate the potential functional significance of the cytoplasmic droplet. This purpose is achieved by demonstrating the presence of lectin binding saccharides D-galactose and N-acetyl-D-galactosamine. Furthermore, we attempt to demonstrate the ability of these saccular elements to glycosylate exogenous and endogenous acceptors. Capability of glycosylation by the cytoplasmic droplet saccular elements infers a potential biological role for these structures. The significance of the cytoplasmic droplet relevant to spermatozoa maturation will be discussed.

LITERATURE REVIEW

LITERATURE REVIEW

I FUNCTIONS OF MAMMALIAN EPIDIDYMIS

The epididymis (Greek adjacent to the testis) is a single, highly convoluted duct continuous with the ductuli efferentes, which are a series of tubules that arise from the rete testis. Its length varies from 3 to 4 m to 80 m in horses (Maneely, 1959). Hammar (1897), who characterized the organ into four distinct regions based on the histological features of the lining epithelium, provided one of the earliest studies on the morphology of mammalian epididymis. Such findings have been confirmed (Maneely, 1955, Reid & Cleland 1957, Nicander, 1957, 1958) and modified by subsequent investigators (Nicander and Glover, 1973, Hoffer and Karnovsky, 1981). The epididymal duct is now generally accepted as being divisible into the initial segments, caput (head), corpus (body), and cauda (tail). Extending from the epididymis is a straight tube, the vas deferens, which is a muscular tube connecting with the urethra that empties to the outside of the body.

Morphological studies at the light microscopic level revealed that the luminal surface of the duct is lined by pseudostratified epithelial cells resting on a basement membrane, which is surrounded by a circular layer of smooth muscle and connective tissue with a rich capillary network. Five different cell types, namely, principal, narrow, clear, basal and halo cells, have been described in the epithelium of the different regions of the epididymis. With the exception of the narrow cells, which are present exclusively in the initial segment, all other cell types are found throughout all regions of the epididymis, although in different proportion (see Table A)

Table A. Relative proportion of different cell types in different regions of the rat epididymis
(Data from Reid and Cleland, 1957)

Regions of epididymis	Cell types				
	Principal	Narrow	Clear	Basal	Halo
Initial segment	80%	3%	0%	12%	5%
Caput	77%	0%	5%	11%	7%
Corpus	69%	0%	6%	21%	4%
Cauda	65%	0%	10%	21%	4%

The epididymis is an androgen-dependent organ (Orgebin-Crist et al., 1975, Majumder and Turkington, 1976, Brooks, 1981a). Androgen receptors on epididymal cells have been identified and studied (Hansson et al., 1974). The synthesis of various epididymal proteins (deLarminat et al., 1978, Jones et al., 1981, Moore et al., 1990) and metabolic enzymes of epithelial cells (Brooks, 1981b) is stimulated and regulated by testosterone. Some of the proteins identified are chromosomal acidic proteins related to differentiation of epididymal cells at puberty (Kadohama and Turkington, 1974). Regulations of epididymal protein synthesis by androgens both at the levels of DNA synthesis (Majumder and Turkington, 1976) and translation of the corresponding mRNA (Brooks, 1987a) have been described.

1) ENDOCYTIC ACTIVITIES IN THE EPIDIDYMIS

Many investigators have shown that the epithelium of the epididymis has the ability to absorb a large volume of fluid entering from the rete testis and to internalize particulate matter and proteins found in the luminal fluid. These activities not only concentrate spermatozoa in the epididymal lumen (8-fold in caput and 20-fold in cauda epididymidis) (Turner et al., 1984), but also modify the environment in which the spermatozoa are exposed to. The mechanism of water absorption in rat epididymis involves passive diffusion following such driving molecules as sodium (in cauda epididymidis) and chloride (in caput epididymidis) (Wong et al., 1978). The transport of these ions is also dependent on androgens (Wong and Yeung, 1977).

Using tracer studies with non-specific markers, Hoffer et al. (1973) observed their presence at increasing time intervals in coated pits and vesicles, multivesicular bodies, and finally in lysosomes of the principal cells of the initial segment. Similar pathways for principal cells of the caput and cauda epididymidis have also been described (Friend, 1969, Moore and Bedford, 1979). The clear cell is another cell type of the epididymal epithelium that is well characterized for its active endocytosis (Moore and Bedford, 1979a, b). Using the adsorptive tracer cationic ferritin in the cauda epididymis, Hermo et al. (1988) demonstrated the presence of the tracer sequentially with time within large coated and uncoated pits and vesicles, numerous

small-sized uncoated vesicles, endosomes, pale and dense multivesicular bodies and finally in secondary lysosomes. The nature of the proteins found normally in the lumen and taken up by the epithelial cells is largely unknown. There is evidence that androgen-binding protein is taken up by principal cells primarily in the efferent ducts and proximal segment of rat caput epididymidis (Attramadal et al., 1981, Pelliniemi et al., 1981). Other proteins such as α -2-macroglobulin and transferrin are also taken up by rat caput principal cells via receptor-mediated endocytosis. However, while both of the latter molecules were shown to be taken up initially in coated pits and vesicles, and subsequently internalized and sequestered within endosomes and multivesicular bodies, only α -2 macroglobulin, like the nonspecific tracers, was seen in lysosomes, while transferrin was reported to be recycled into the epididymal lumen (Djakiew et al., 1984, 1985).

Besides absorption of fluid and other particulate matters, the epididymal epithelium further modifies the epididymal fluid by secretion of proteins and other substances into the lumen. The osmolality (D'Addario et al., 1980) and chemical composition (Brooks, 1979, Howards et al., 1979, Lechene, 1982, Legault et al., 1979) of the fluid secreted by the epididymis vary from one segment to another. Several investigators have demonstrated segmental increases in concentrations of many intraluminal substances, including ions such as potassium and phosphorus, electrolytes (Levine and March, 1971, Jenkins et al., 1980), small organic molecules such as carnitine (Marquis and Fritz, 1965), inositol (Hinton et al., 1980) and glycerylphosphorylcholine (Bjerve and Reitan, 1978) as well as numerous proteins and glycoproteins (Lea et al., 1978, Moore, 1980, Flickinger, 1981, Brooks, 1983).

2) EXOCYTIC ACTIVITIES OF THE EPIDIDYIMIDIS

The role of epididymal epithelial cells in protein secretion has long been speculated. Various studies in the 70's demonstrated that the electrophoretic pattern of protein bands from epididymal fluid differs not only from plasma but also among different epididymal regions (Barker and Amann, 1971, Amann et al., 1973, Jones, 1974, Koskimies and Kormanio, 1975, Dacheux and Voglmayr, 1983). Using

[³H]-galactose as marker in light microscopic radioautography, Neutra and Leblond (1966) provided the first convincing evidence indicating glycoprotein synthesis and secretion in epididymal principal cells. Their findings were substantiated in later studies by others (Kopecny and Peck, 1977). Incorporation of labeled amino acids into proteins by principal cells of the epididymis has been well documented (Vendrel and Durhat 1968, Kopecny, 1971, Kanka and Kopecny, 1977). Using electron microscopy and radioautography, Flickinger (1981, 1983, 1985) further demonstrated that subsequent to protein synthesis in rough endoplasmic reticulum, labeled amino acids appeared over the Golgi apparatus, and later over the apical cell surface and finally inside the epididymal lumen. Extensive efforts have since been devoted to the study of various glycoproteins synthesized and secreted by epididymal epithelium.

Principal cells in a specific segment of the distal caput and proximal corpus epididymis in mouse, defined histologically by Pavlok (1974), were shown to secrete a protein termed sperm maturation antigen number four (SMA 4) (Vernon et al., 1982). Using immunocytochemistry with different specific antisera, secretion of various other proteins by principal cells in equivalent regions of the epididymis was also found in different mammalian species. These various proteins include the epididymal-specific proteins in rabbit and hamster (Moore, 1980) and, in rat epididymis, a 37 kD epididymal sialoprotein (Faye et al., 1980), a 33 kD acidic epididymal glycoprotein (Lea et al., 1978), and a specific epididymal protein (SEP) (Kohane et al., 1980). Other examples of proteins secreted by the epididymis in similar regions include the acrosome stabilizing factor (Thomas et al., 1984), bovine forward motility protein (Brandt et al., 1978), and secretory proteins of human epididymis (Tezon et al., 1985).

A group of proteins, known as the mouse epididymal proteins (MEP), secreted by the principal cells, were recently characterized and immunolocalized (Rankin et al., 1992). A member of these, MEP 7, a 29 kD glycoprotein, is secreted in the distal caput, corpus and cauda epididymidis. Rankin et al. (1992) suggested that this protein not only has properties similar to several other epididymal proteins in other animal

species, including CP27 (Flickinger et al., 1988), PES (Fournier-Delpech et al., 1973), protein IV (Jones et al., 1980) and a 32 kD protein (Wong and Isang, 1982), but probably is a homologue of the rat acidic epididymal glycoprotein (AEG) (Lea et al., 1978) and proteins D and E (Brooks and Higgins, 1980). MEP 10, a 18 kD protein, is immunolocalized within principal cells at the junction of the distal caput and corpus. It is similar to proteins B and C isolated from rat epididymis by Brooks and Higgins (1980) and epididymal binding proteins (FBP) 1 and 2 from rat caudal fluid (Newcomer and Ong, 1990). MEP 9 is a 25 kD glycoprotein immunolocalized within all principal cells of the distal caput and within only some principal cells of the corpus and cauda epididymis (Rankin et al., 1992, Vierula et al., 1992). Epididymal proteins of similar molecular mass include a 24 kD protein secreted by the mid and distal caput epididymidis of the mouse (Jimenez et al., 1990), a 26 kD protein found in the cauda region of the mouse epididymis (Murphy and Carroll, 1987), and a rat 26 kD sperm binding protein present caudal fluid and on caudal spermatozoa but not in the caput epididymidis (Olson et al., 1987). All of these proteins differ in their distribution and biochemical properties from MEP 9 (Rankin et al., 1992). A recent study pointing to a similarity in the immunolocalization of MEP 9 with the Yo subunit of glutathione S-transferase (Veri et al., 1993), suggests that MEP 9 is the mouse homologue of this subunit of glutathione S-transferase.

All of these studies as well as others not only indicate the exocytic function of principal cells but also demonstrate the regional variation of protein secretion in different segments along the length of the epididymis. Presumably each successive segment of the epididymis is to fulfill some essential and perhaps cumulative role in the maturation of the spermatozoa. Resolving the role of each secretory proteins and the identification of the regulatory factors involved in its secretion is crucial to understanding the maturational process of spermatozoa.

Many of the secreted proteins become associated with spermatozoa in specific regions (see section II 2 a), while some others appears to be reabsorbed in more distal regions of the epididymis. An examples of the latter include a 37 kD SP protein, secreted by the epithelial cells of the caput and corpus epididymidis, which is

reabsorbed by the epithelial cells, possibly clear cells, in the cauda region (Faye et al, 1980) Flickinger et al (1988) indicated that a 27 kD glycoprotein (CP 27), secreted by the distal caput-proximal corpus epididymidis, is reabsorbed in the distal corpus and cauda epididymidis by clear cells

3) SIGNIFICANCE OF THE EPIDIDYMIS IN SPERMATOZOON MATURATION

In mammalian species, spermatozoa shed from the testis are immature, immotile and incapable of fertilizing the ovum. During their transit through the epididymis, they go through various morphological and functional changes that confer to them the ability to ascend the female tract, to undergo the acrosome reaction, to penetrate the zona pellucida and to effect a successful fertilization. The microenvironment of epididymal lumen provided by the epithelial cells via various endocytic or exocytic process may have a crucial role in rendering spermatozoa fertile. The recent findings of a regional variation of protein secretion in epididymis, as described above, further illustrate the concept that perhaps each successive segment of the epididymis is to fulfill some essential and cumulative role in the maturation of the spermatozoa. Such a concept was first introduced by Von Ebner (1888) and by Van der Stricht (1893) and remains well accepted to date. Among the most convincing evidence of such a biological role of the epididymis is the increase in the ability of immature sperm to bind to the zona pellucida and to fertilize oocytes after incubation with purified epididymal proteins (hamster Cusanicu et al, 1984, Gonzales Echeverria et al, 1984, Moore and Hartman, 1986, rat Orgebin-Crist and Fournier-Delpech, 1982, Cusanicu et al, 1984). The identity of the mediators of this effect and their mode of action is still unknown.

Orgebin-Crist (1969) pointed out that convincing evidence is still lacking as to whether the factors governing the maturation process of spermatozoa are intrinsic to the spermatozoa themselves and just require time, or whether spermatozoa must pass through most of the epididymis in order to mature. Silber (1989a) performed a series of studies to investigate the fertility of a hundred and thirty-nine human subjects with bilateral epididymal obstruction who underwent vasoepididymostomy performed by microsurgery. Passage of spermatozoa through different regions of the epididymis

is thus bypassed. In a group with vasoepididymostomy to the caput epididymidis (i.e. epididymal tubule at the end of caput epididymis is diverted to the vas deferens, bypassing the corpus and cauda epididymis), the pregnancy rate by natural intercourse was up to 43%, while in the group where anastomosis was performed in the mid corpus epididymidis (i.e. spermatozoa diverted from mid corpus epididymis to vas deferens), the pregnancy rate was 72%, with a mean time to conception of six months. Clinical case reports have demonstrated that it is even possible, in some circumstances, for spermatozoa which have never traversed any length of epididymis to fertilize the human egg (Silber, 1988). In addition, successful pregnancy from the aspiration of proximal caput epididymal spermatozoa combined with in vitro fertilization and zygote intra-fallopian transfer in cases of irreparable obstruction (Silber et al., 1988) gives further evidence that transit through the epididymis is not a mandatory requirement for fertilization. Although such evidence was based on case reports, it nonetheless indicates the possibility that, at least in human, epididymal transit may not be essential to spermatozoon development and fertility.

II CURRENT CONCEPTS OF EPIDIDYMAL MATURATION OF SPERMATOOZA

In a series of studies performed in the 60's and 70's (Blandau and Rumery, 1964, Bedford, 1966, Orgebin-Crist, 1967, Horan and Bedford, 1972, Pavlok, 1974, Waiters, 1979) where spermatozoa isolated from different regions of the excurrent ductal systems, including the testes, efferent duct, caput, corpus and cauda epididymidis and vas deferens, were tested for their ability to fertilize ova, it was found that spermatozoa isolated from the seminiferous tubules of testes are virtually immotile and incapable of fertilization. More interesting was the finding that the percentage of the population of sperm that are motile and capable of fertilization gradually increases as they move distally along the excurrent duct system. Such observations hold true amongst all species of mammalian studied. The process through which spermatozoa obtain their ability to fertilize is termed epididymal maturation, for it happens essentially during their transit through the epididymis.

The exact site of the epididymis where the spermatozoa begin to acquire their fertilizing ability varies from species to species. In some species (e.g., the boar), it is the distal segment of caput epididymis, while in others (e.g., the rat) it is the distal segment of the corpus epididymis (Dacheux and Paquignon, 1980). It is unlikely that all the spermatozoa gain their fertilizing capacity simultaneously. Some spermatozoa apparently become fertile much faster (i.e., in a more proximal region of the epididymis) than other (reviewed by Yanagimachi, 1988). Generally speaking, however, maturation of the great majority of spermatozoa of different mammalian species is believed to be completed by the time they reach the distal caudal segment of the epididymis.

Studying the mechanism and regulation of epididymal maturation of spermatozoa has long been one of the biggest challenges to spermatologists. The clinical implications of understanding sperm maturation lie in solving problems of infertility in human as well as other animal species. Furthermore, information as such would help devising methods that can reliably interfere or arrest such maturation process, which can thus be used for contraception in males.

Various studies have been performed to shed light on the processes involved in sperm maturation. Morphological changes of spermatozoa in various species during their epididymal transit have been well documented (Fawcett & Philips, 1969, Bedford, 1974). Most changes appear to involve various domains of the sperm head including the acrosome (Leblond & Clermont, 1952b, Fawcett and Hollenberg, 1963), the subacrosomal space (Fawcett and Hollenberg, 1963), and postacrosomal sheath (Pederson, 1972). With the aid of electron microscopy in conjunction with biotechnology, modifications in spermatozoa during epididymal transit have been studied at the molecular level (Hoffer et al., 1981).

1) ACQUIREMENT OF MOTILITY OF SPERMATOZOA

The proportion of motile spermatozoa and the duration of their motility *in vitro* increase in the distal segments of the epididymis. Although some motile spermatozoa are found in the caput epididymidis, in most mammals these spermatozoa have only a vibratory or slow and ineffective beat that often results in circular swimming patterns without any sign of forward progression (Bedford, 1975). Assessment of sperm motility using photographic measurements revealed that progressive motility of spermatozoa in most species appeared first in the corpus epididymidis (reviewed by Eddy, 1988).

The initiation and regulation of sperm motility have been shown to involve a variety of factors (Majumder et al., 1990). A unifying hypothesis for how these factors interact to accomplish their roles in rendering sperm motile is lacking. Amongst the various factors involved, cyclic adenosine monophosphate (cAMP) is the best studied. It is well established that cAMP levels increase in sperm during epididymal transit (Hoskins et al., 1974, Amann et al., 1982, Atherton et al., 1985) and exogenous cAMP has been shown to stimulate sperm motility (Garbers et al., 1971, Hoskins et al., 1978). In rats, the level of a cAMP-dependent protein kinase is higher in cauda epididymal spermatozoa than those in caput epididymidis (Atherton et al., 1986). The major role of cAMP in sperm is probably to mediate cAMP-dependent phosphorylation of proteins that are essential for both initiation of

motility in the epididymis and maintenance of motility (Garbers & Kopf, 1980, Garbers et al., 1982, Hoskins 1973)

The profile of rat sperm protein phosphorylation undergoes marked alteration during epididymal maturation (Chulovatnatol et al., 1982) Amongst the many candidates of phosphoproteins is a heat-stable glycoprotein of epididymal origin known as forward motility protein (Acott & Hoskins, 1978, Hoskins et al., 1978) It has been isolated from bovine epididymal and seminal plasma and it appears to work in concert with intracellular cAMP in the initiation of forward motility in the immature bovine caput epididymal spermatozoon Similar proteins with different molecular weights have been found in other species where they are thought to be involved in the development of sperm motility as the sperm moves from caput to cauda epididymidis Other phosphoproteins reported to involve in the initiation of sperm motility include a 55 kD protein in bull sperm (Brandt & Hoskins, 1980) and in dog and human a 56 kD soluble protein, known as axokinin, which was present in extracts of immature testis, indicating that it was synthesized during spermatogenesis (Tash et al., 1984)

Transfer from epididymal fluid to spermatozoa of such substances as the carnitine (Carsillas, 1973, Hinton et al., 1981, Casillas and Chaipayungpan, 1982, Inskeep and Hammerstedt, 1982), glycerophosphocholine (Infante and Huszagh, 1985), immobilin (Usselman and Cone, 1983), sperm-motility inhibiting factor (Turner and Giles, 1982), sperm-motility quiescence factor (Carr and Acott, 1984), during epididymal maturation is believed to be important in rendering or regulating mobility to spermatozoa There are other mammalian sperm surface molecules that may influence sperm transport In the presence of antisperm antibodies, these surface molecules may interact with the antibodies to cause spermatozoa with vigorous motility to resist forward progression and thus prevent fertilization (Bronson et al., 1985) In addition p-chloromercuriphenylsulfonic acid (PCMPS), a thiol reagent that does not penetrate the sperm plasma membrane, strongly inhibits goat sperm flagella motility (Majumder et al., 1988), this inhibition of motility cannot be reversed with the addition of excess of 2-mercaptoethanol or dithiothreitol (Majumder and Chaudhuri,

1984) Thiol-containing proteins that may be essential for flagella motility may thus be localized on the sperm outer surface. The identity of these macromolecules on the external cell surface that may serve as the molecular determinants of sperm motility is still unknown.

2) MODIFICATION OF SPERMATOZOA PLASMA MEMBRANE COMPOSITION DURING EPIDIDYMAL MATURATION

It has long been speculated that the development of progressive motility and fertilizing ability by spermatozoa depends on modifications occurring during their passage through the epididymis (for reviews, see Bedford, 1975, Hamilton, 1975, Orgebin-Crist et al., 1975, 1981, Olson, 1984, Austin, 1985). Many surface alterations on spermatozoa result from interactions with the surrounding epididymal fluid. These modifications of externally located macromolecules are often associated with restricted surface domains such as the plasma membrane covering the sperm head (Courstens et al., 1982, Brooks and Tiver, 1983, Rifkin and Olson, 1984, Tezon et al., 1985). This suggests the specificity of their functions in motility or gamete recognition events of fertilization (Fawcett, 1975, Koehler, 1981, Olson and Orgebin-Crist, 1982, Lambert and Van Le, 1984, Primakoff et al., 1985, 1987, Eddy, 1988, Yanagimachi, 1988, Leyton and Saling, 1989, Phelps et al., 1990). It is important to note that the modification in plasma membrane over the tail regions during sperm maturation have also been reported (Brown et al., 1983, Brooks, 1985, Tezon et al., 1985, Olson et al., 1986). It thus appears that the entire sperm plasma membrane, from the anterior end of the sperm head to the distal end of the sperm tail, is likely to change its chemical characteristics during epididymal maturation. Several authors (Hamilton, 1975, Yanagimachi, 1981, Eddy et al., 1985) proposed three possible mechanisms whereby sperm surface is modified during epididymal maturation: i) direct addition of new components to the sperm surface, ii) loss of sperm-surface components, or iii) unmasking or modification of pre-existing sperm surface moieties.

a) Proteins & Glycoproteins

Modifications of proteins and glycoproteins on epididymal spermatozoa have been studied most extensively in rat, although such modifications in other species have also been documented. It is thought that some of these proteins have a functional role in the development of fertilizing ability. In the rat, there has been several studies (Brooks, 1982, Faye et al., 1980, Lea et al., 1978, Wong and Tsang, 1982) dealing with a group of similar proteins with a molecular mass in the range of 32 kD. It has been suggested that a protein of this group might have a role in the binding of the spermatozoa to the zona pellucida (Orgebin-Crist et al., 1975). These studies prompted a further investigation by Brown et al. (1983) to compare changes in plasma membrane glycoproteins of rat testicular sperm and of cauda epididymal spermatozoa. They found that unique to the plasma membrane of cauda epididymal spermatozoa were 13.5, 32, 47, 84 and 150 kD glycoproteins of which the 32 kD protein was the most conspicuous.

A group of researchers (Olson and Danzo, 1981, Olson et al., 1987) demonstrated, by lactoperoxidase-catalyzed radio-iodination of the rat epididymal spermatozoon surface, that a 26 kD glycoprotein appears on the plasma membrane of cauda sperm but not on caput sperm. Immunohistochemical studies using monospecific antibodies obtained against this protein showed that this antigen was not detectable on caput sperm but first appeared on sperm from the proximal corpus epididymidis.

In another series of studies, a 30 kD (protein "D") and a 32 kD (protein "E") protein isolated and purified from rat caput epididymidis were found to be absent from testicular spermatozoa but present on cauda spermatozoa (Brooks and Higgins, 1980, Brooks 1981 a & b). Antisera to protein "D" and "E" respectively showed that they concentrate mainly on the head of sperm (Brooks and Tiver, 1983).

Several spermatozoon maturation-dependent membrane glycoproteins, ranging from 19 to 97 kD have also been identified in epididymal luminal fluid (Lea et al., 1978, Olson and Danzo, 1981, Jones and Brown, 1982, Wong and Tsang, 1982, Brown et al., 1983, Brooks, 1985, Hamilton et al., 1986, Iusem et al., 1989, Srivastava and Olson, 1991). Such findings support the hypothesis that these

glycoproteins are secreted by the epididymal epithelium and subsequently bind the sperm surface (Srivastava and Olson, 1991). There are many well-established examples regarding this possibility: acidic epididymal glycoprotein (Lea et al., 1978), forward motility protein (Brandt et al., 1978, Acott and Hoskins, 1978, 1981), acrosome stabilizing factor protein (Thomas et al., 1984), His-proteins (Rifkin and Olson, 1985), SGP-2 (Sylvester et al., 1984, 1991, Hermo et al., 1991a), and androgen dependent proteins (Jones et al., 1980, Brown et al., 1983, Brooks et al., 1986, Brooks, 1987a & b, Moore et al., 1990) have all been shown to be secreted by various regions of the epididymal epithelium and then become associated with the sperm surface.

Klinefelter and Hamilton (1985) showed by perfusion organ culture of proximal and distal rat caput epididymal tubules that at least five polypeptides labeled with [³⁵S]-methionine are synthesized and secreted by these cultured tubules and become associated with luminal sperm. Using retrograde perfusion of the superior and inferior epididymal arteries with [³⁵S]-methionine, Vreeburg et al. (1993) devised a method to label epididymal proteins *in vivo* and to study the fate of the proteins after secretion, especially whether they eventually bind to spermatozoa. These authors reported binding of three epididymal proteins (25 kD, 30 kD and 32 kD) on rat epididymal spermatozoa. The significance of these proteins, however, remains to be elucidated. Up to this point, the mechanisms of association of these secreted proteins with the sperm membrane remain questions and it is not clear whether these proteins are integral or surface components of the plasma membrane.

Epididymal secreted proteins may not account for all of the new glycoproteins implicated to be acquired by spermatozoa plasma membrane during epididymal maturation. In view of the low rates of protein synthesis exhibited by mammalian spermatozoon after spermiation, the gradual modification of its surface-glycoprotein content is unlikely to be due to *de novo* glycoprotein synthesis with subsequent incorporation to its plasma membrane. Another possibility is that through some unmasking or alteration events, glycoproteins (and other surface moieties) are modified, thereby rendering maturity of spermatozoa as they pass through the

epididymal lumen Extrinsic glycosylation of pre-existing sperm membrane proteins is an example of how sperm surface components may be modified (see section c)

b) Lipids

Lipid is a major constituent of the plasma membrane and plays an important role in constituting the membrane structure by modulating the fluidity of the biomembranes. Consequently, the lipid constituents have a profound influence on the regulation of membrane functions. The cholesterol/phospholipid ratio and the concentration of phosphatidylserine, phosphatidylethanolamine, cardiolipin, and ethanolamine plasmalogen has been reported to decrease in ram spermatozoa during epididymal maturation (Quinn and White, 1967, Scott et al, 1967). A decrease in the content of sperm total lipid during transit of spermatozoa through epididymis has also been reported in boars (Evans and Setchell, 1979), bulls (Quinn and White, 1967), rams (Poulos et al, 1973, Dacheux, 1977), and rats (Dawson & Scott, 1964). This decrease in the lipid content of spermatozoon as a consequence of epididymal maturity may be due to its utilization as an energy source. Reports on changes of sperm lipid composition during epididymal maturation are mostly based on lipid extracts of whole sperm cells (Scott, T W, et al, 1967, Evans & Setchell, 1979, Quinn & White, 1967, Poulos et al, 1973). Since the plasma membrane represents less than 35% of the total cellular lipids (Lunstra et al, 1974), the data obtained with whole cell lipid analysis are only of nominal use in explaining plasma membrane features in relation to its lipid composition (Rana et al, 1991).

Data on lipid composition of membranes derived from spermatozoa obtained from boar (Nikolopoulou et al, 1985) and ram (Parks & Hammerstedt, 1985) and goat (Rana et al, 1991) showed some similarities and dissimilarities in respect of their alteration during sperm maturation. Although phosphatidylcholine and phosphatidylethanolamine were the major phospholipids in the sperm membrane of all the species, marked differences were noted in their maturation associated changes. The cholesterol/phospholipid ratio increased during the epididymal maturation of ram (Parks and Hammerstedt, 1985) and goat sperm (Rana et al, 1991), but it decreased

in boar sperm (Nikolopoulou et al., 1985). In addition, although the nature of glycolipids were different in goat and boar sperm membrane, their contents in both species decrease markedly during the maturation of the male gametes.

Recent studies have shown that the lipid-phase fluidity of the sperm plasma membrane decreases significantly during the epididymal maturation of the male gametes (Rana and Majumder, 1990). It is well documented that the membrane microviscosity is greatly influenced by cholesterol/phospholipid and saturated/unsaturated fatty acids ratios of the lipids (Stubbs and Smith, 1984). It thus appears that the observed marked increases in the above-mentioned ratios of lipids and fatty acids during the epididymal sperm maturity is primarily responsible for the maturation-dependent changes in the sperm membrane fluidity. It has been speculated that the altered fluid state of the membrane consequent upon maturation may have an important role in the regulation of membrane function with special reference to ion transport, activity of membrane-bound enzymes, masking and unmasking of cell surface macromolecules (Stubbs and Smith, 1984, Rana and Majumder, 1990). At present, however, little is known regarding the biological significance of the differential maturation-associated changes of the sperm membrane lipids in the different species.

c) Saccharides

One of the few modifications of spermatozoa plasma membrane that correlates with the steady increase in the ability of the sperm head to adhere to the egg zona pellucida as the spermatozoa pass through different regions of the epididymis (Orgebin-Crist and Fournier-Delpech, 1982, Sailing, 1982, Funaki et al., 1983, Peterson et al., 1986) is the gradual acquisition of a negative surface charge (Bedford, 1963, Cooper and Bedford, 1971, Bedford et al., 1973, Bedford, 1979, Moore, 1979, Holt, 1980, Yanagimachi, 1988). Such an increase in negative surface charge was found to coincide with the increase in the amount of lectin binding sites (Nicholson et al., 1977, Olson and Danzo, 1981, Hamilton and Gould, 1982, Hamilton et al., 1986). Holt (1980) quantified and compared the density of colloidal iron hydroxide

(CIH) binding in ram caput and cauda epididymal spermatozoa and noticed a four-fold higher CIH-binding in cauda spermatozoa. Similar results had been reported in other mammalian species (Cooper and Bedford, 1971, Yanagimachi et al, 1972) previously. The additional finding that the CIH-binding capacity of cauda epididymal spermatozoa was almost totally abolished by neuraminidase treatment led Holt (1980) to conclude that the increase in the net negative surface charge on spermatozoa during epididymal transit is attributable to the acquisition of sialic acid groups on the sperm surface. Furthermore, since Ficoll washing of epididymal sperm did not affect the capacity of CIH-binding, he concluded that the sialic acid carbohydrate groups were attached to proteins which were integral components of the plasma membrane.

In an attempt to identify sperm plasma membrane proteins which acquire carbohydrate residues during epididymal maturation, Olson and Hamilton (1978) used a galactose-oxidase-tritium-borohydride procedure to radioactively label sperm surface glycoproteins possessing terminal D-galactose or N-acetyl-D-galactosamine residues. They found that cauda epididymal spermatozoa of rats possess a 37 kD glycoprotein labeled with both carbohydrate residues while no such protein was detected on caput or corpus epididymal spermatozoa.

In a recent *in vitro* study of glycosylation of spermatozoa, Tulsiani et al (1993) showed that the incorporation of [¹⁴C] fucose was the highest in plasma membranes of spermatozoa from the distal caput. In addition, it was determined that the fucose label was incorporated into an 86 kD plasma membrane protein. Boldt et al (1988, 1989) suggested a role for L-fucose on sperm surface components in sperm-egg fusion in the mouse. This evidence is based on observations including the following: 1) that L-fucose and its polymer (fucoidin and ascophallan), when added to a mixture of sperm and zona-free eggs, have a dose-dependent adverse effect on sperm-egg fusion, and 2) that treatment of capacitated spermatozoa with alpha-L-fucosidase (an exo-enzyme that cleaves terminal alpha-linked fucosyl residues) caused inhibition of sperm-egg fusion.

Extrinsic glycosylation of pre-existing sperm membrane proteins has been

hypothesized as a mechanism whereby spermatozoa plasma membrane is modified. The presence of various glycosylation activities in the contents of various epididymal regions has been well documented. Various glycosyltransferases and glycosidases and some of their regulatory proteins have been identified in epididymal fluid (glycosyltransferases by Letts et al., 1974, Reddy et al., 1976, Tadolini et al., 1977, Bernal et al., 1980, Hamilton, 1980, Hamilton and Gould, 1982, Jones & Brown, 1982, Cossu and Boitani, 1984, Iusem et al., 1984, Takase and Ebner, 1984, Klinefelter and Hamilton, 1985, glycosidases by Kemp and Killian, 1978, Grandmont et al., 1983, Chapman and Killian, 1984, Skudlarek and Orgebin-Christ, 1986).

While activities of some glycosylation enzymes such as androgen-dependent glucosyl and mannosyl transferases (Iusem et al., 1984) could be found throughout the whole epididymis in rat, there has been studies showing that the enrichment of the activities of other glycosylation enzymes differ in different segments of the epididymis. For example, Beta-N- glucosaminidase, Beta-N-acetyl-galactosaminidase and Beta-N- galactosidase activities have been found to be the highest in corpus epididymidis (Chapman and Killian, 1984). Sialyltransferase (N-acetylneuraminyl transferase) activity is higher in caput than cauda epididymidis (Bernal et al., 1980), although some sialoglycoproteins in the epididymal fluids are produced only in cauda epididymidis (Toowicharanout and Chulavatnatol, 1983). Hamilton (1980) measured the enzymatic activity of N- acetylglucosamine galactosyltransferase in fluids from rat rete testis and epididymis. By cannulation of the rete and exudation of epididymal fluid, Hamilton (1980) showed that the specific enzyme activity of galactosyltransferase in the luminal fluid was significantly higher (> 5 folds) in the rete than in the caput epididymidis, the enzyme activity per unit volume of the fluid then decreased abruptly in the distal cauda. This decrease in galactosyltransferase activity led Hamilton (1980) to suggest that this enzyme has a relationship to spermatozoon maturation. An equally important finding was that this enzyme, which was shown to be more kinetically analogous to milk galactosyltransferase than to serum galactosyltransferase, appears to be synthesized and secreted by the testis.

It is important to note that not all glycosylation enzymes are of epididymal origin.

For example, galactosyltransferase found in epididymal fluid appears to be produced in the testis and concentrated in the caput epididymidis (Hamilton, 1980). In addition, some authors reported galactosyltransferase activity on the sperm surface (Durr et al, 1977, Shur and Bennett, 1979, Lopez et al, 1985) which may be involved in sperm-surface modifications during epididymal maturation. Tulsiani et al. (1993) reported 80-90% of four glycosyltransferase (sialyltransferase, fucosyltransferase, galactosyltransferase, and N-acetyl glucosaminyltransferase) activities to be present in soluble form in the epididymal fluid. The remaining 10-20% of total enzymes activities is associated with spermatozoa fractions. Tulsiani et al. (1993) quantified the activities of four glycosyltransferase (sialyltransferase, fucosyltransferase, galactosyltransferase, and N-acetyl glucosaminyltransferase) in epididymal fluid obtained from five representative regions of the epididymis. On measuring the sperm-associated glycosyltransferase activities per 10^6 spermatozoa, it was found that only sialyltransferase and fucosyltransferase activities displayed a gradual reduction as spermatozoa moved from the rete testis to the distal cauda epididymidis. They further suggested that the higher levels of the two enzymes on caput spermatozoa could be due to their binding to the endogenous sugar acceptor molecules on the sperm surface, and subsequent release following sequential sialylation and fucosylation of the molecules in the proximal and distal caput spermatozoa, where activities of sialyltransferase and fucosyltransferase, respectively, were found to be highest.

d) Other macro-molecules

With the advantage of their specificity, monoclonal antibodies have been used to examine sperm-surface changes during epididymal maturation. Studies using the immunological approaches have complemented biochemical studies in identifying numerous macro molecules on sperm surface that correlate with sperm maturation in epididymis.

Two rat sperm-surface antigens that first appear in the caput epididymis were identified (Gaunt et al, 1983) one was present on the postacrosomal portion of the

head (identified with monoclonal antibody 1B6), and the other was uniformly distributed over the entire sperm surface (identified with antibody 2D6). The latter antigen was susceptible to antibody-induced patching and was observed to be inserted into the egg surface upon fertilization (Lea et al., 1978).

Two antigens were also identified on hamster spermatozoa that were modified during maturation (Cameo and Blaquier, 1976). One was over the head at an apparently higher concentration and on a greater percentage of cauda epididymal sperm than on testicular sperm (identified by antibody HM 3.1). The other was over the entire tail and appeared to be present in higher concentrations on corpus sperm than on caput or cauda sperm (identified by antibody HM 5.8). Antibody HM 3.1 blocked fertilization *in vitro*, whereas HM 5.8 reduced fertilization *in vitro* due to sperm agglutination.

In the mouse, a 28 kD antigen (identified with monoclonal antibody 1B3) was present over the entire surface of spermatozoa from the testis or caput epididymidis, but was present only on the tip of the head of sperm from the cauda epididymidis (Gaunt, 1982). Using monoclonal antibodies in mouse, other studies identified four sperm-maturation antigens (SMA) which were restricted over regions of the anterior acrosome (SMA 1), posterior acrosome and midpiece (SMA 2), whole head (SMA 3), and whole tail (SMA 4) (Feuchter, 1981). SMA 4 was secreted by the epithelium in the distal-caput-proximal-corpus region of the epididymis as an 85 kD component and was cleaved to a 54 kD component upon attachment to the surface of the flagellum (Vernon et al., 1982; Vernon et al., 1985; Vernon et al., 1987).

3) POTENTIAL ROLE OF GALACTOSYLTRANSFERASE IN BINDING ZONA PELLUCIDA

We have discussed the importance of carbohydrates on macromolecules and how they can be modified by glycotransferases and/or glycohydrolases. Recently, the potential role of a glycosyltransferase, namely galactosyltransferase, on sperm membrane as a receptor for zona pellucida binding has received a lot of attention. It has been proposed that gamete recognition is dependent on sperm surface

carbohydrate binding proteins with high affinity and specificity to complex glycoconjugates of the egg coat (see review by Macek & Shur, 1988) It is not surprising that interactions between complementary cell surface proteins and glycoconjugates participate in fertilization, since a number of somatic cells appear to utilize a similar paradigm for intercellular recognition (Damsky et al , 1984, Ivatt, 1984)

Evidence supporting such a functional role of galactosyltransferase include genetic studies in mouse It was found that in a strain of mice that have a genetic predisposition for increased fertilizing ability also possess an elevated sperm-surface galactosyltransferase activity (Shur and Bennett, 1979) However, sperm from recombinant strains of these mice did not show elevated galactosyltransferase activity or increased fertilizing ability (Shur and Hall, 1982a) Several investigators reported that certain reagents that perturb sperm galactosyltransferase inhibit sperm-egg binding Competitive substrates for the enzyme, alpha-lactalbumin, and other enzyme substrate analogs inhibited sperm binding to the zona pellucida (Shur and Hall, 1982a & b) Affinity-purified galactosyltransferase and UDP-galactose produced a dose-dependent inhibition of sperm binding to the zona pellucida and caused sperm bound to the zona pellucida to be released, presumably due to competition of binding substrates on the zona pellucida (Lopez et al , 1985) In addition, monospecific antiserum to the enzyme produced a dose-dependent inhibition of sperm binding to the zona pellucida and concomitantly blocked sperm galactosyltransferase activity (Shur and Hall, 1982 a,b, Lopez et al , 1985)

Interestingly, it was suggested that galactosyltransferase is present throughout all stages of spermatogenesis, during which time it redistributes within the plasma membrane from a uniform, diffuse distribution on primary spermatocytes to a restricted domain overlying the dorsal surface of the mature acrosome (Scully et al , 1987) It was, however, not clear why immature sperm that possess surface galactosyltransferase are infertile One plausible explanation was provided by McLaughlin and Shur (1987) who noted the presence of high level of activities of soluble galactosyltransferase and alpha-lactalbumin in epididymal fluid Earlier studies

have shown that both galactosyltransferase and alpha-lactalbumin can inhibit cauda epididymal sperm from binding to the zona pellucida (Shur and Hall, 1982a & b, Lopez et al, 1985) These data led McLaughlin and Shur (1987) to suggest that caput epididymal fluid are able to inhibit the binding of mature cauda epididymal spermatozoa to the egg zona pellucida However, even after removing the inhibitory epididymal secretions by repeated washing and centrifugation, the zona pellucida binding ability of caput epididymal sperm are never as well as cauda epididymal spermatozoa (McLaughlin and Shur, 1987) Thus, as pointed out by these authors, there appear to be factors other than the presence of soluble epididymal inhibitors that regulate the fertility of epididymal spermatozoa

III THE CYTOPLASMIC DROPLETS

The cytoplasm of spermatids in testis undergoes drastic reorganization at the later steps of spermiogenesis. Spermatids begin with a substantial volume of cytoplasm containing various organelles for their metabolism, while spermatozoa collected in the ejaculate contain only a minimal amount of cytoplasm. Between these two stages of maximum and minimum amounts of cytoplasm, the spermatozoa retain a small volume of cytoplasm as they pass along the epididymis. This mass of cytoplasm is termed the cytoplasmic droplet. This structure was first described by Retzius in 1909 in bull and appears to be universal among mammalian species. The cytoplasmic droplet eventually detaches from the sperm tail in the distal part of the epididymis. The process of detachment, described in detail later, involves the movement of the cytoplasmic droplet along the sperm tail. Such a phenomenon is noted in many mammals (Branton and Salisbury, 1947, Nicander, 1956), including human (Hafez and Prasad, 1976), and also in snakes and birds (Bedford, 1979) which, like mammals, also produce sperm with cytoplasmic droplets. The passage of the cytoplasmic droplet is in fact the most prominent morphological change of spermatozoa during their epididymal migration and maturation.

The biological significance of the cytoplasmic droplet remains a mystery among spermatologists and their views towards the potential roles of this organelle are diverse. While some investigators speculated that the droplets play a role in the nourishment of the spermatozoon as it passes through the epididymis (Moniem and Glover, 1972), others held the view that the organelle is devoid of any special functional significance. It has even been suggested that the cytoplasmic droplet may cause damage to maturing spermatozoa and therefore must be removed as spermatozoa mature in the epididymal lumen (Temple-Smith, 1984). Interestingly, infertility appears to be related to the cytoplasmic droplets. Lagerolf (1934) reported that spermatozoa with the droplet retained in their anterior position show a poor fertility and ejaculates containing a high percentage of such spermatozoa are considered to indicate a spermatogenetic disturbance. The association between loss of fertilizing capacity and a high proportion of ejaculated spermatozoa retaining their

droplets was also noted by Cummins and Glover (1968) who used artificial cryptorchidism as a model to study the pathology of the reproductive system. The association between infertility and the retention of the droplet by spermatozoa in ejaculates has also been well documented also in bovines (Barth & Oko, 1990). Various congenital or acquired defects of spermatozoa, such as Daglike defects, distal midpiece reflex and corkscrew defect invariably show retention of cytoplasmic droplets at the site of sperm tail where abnormal morphology is most conspicuous. In addition, the same authors reported that the correlation between the high percentage of droplets surrounding the neck and proximal midpiece region (proximal droplets) of spermatozoa and decrease in semen quality for fertilization (Barth and Oko, 1990). On the other hand, high percentage of droplets surrounding the midpiece just proximal to the annulus (distal droplets) is not considered a serious problem. The cytoplasmic droplet has therefore gained some importance as a morphological indicator of sperm maturation (Bloom and Nicander, 1961, Cummins and Glover, 1970, Cummins, 1973, Bedford, 1978).

1) SPERMATOZOA CYTOPLASM REORGANIZATION IN SPERMATION

The process whereby the spermatid leaves the seminiferous epithelium of testis is termed spermiation. Morphologically, the most conspicuous changes of the late spermatid prior to spermiation is the reorganization of the cytoplasm and its organelles. Such a reorganization process of sperm cytoplasm has been described in several mammalian species in detail by Leblond and Clermont (1952a) to determine the various steps of spermiogenesis.

The cytoplasm of early spermatid shows the well-documented ultrastructure which includes condensed mitochondria, a Golgi apparatus in juxtaposition to the developing acrosomic system, a vesicular endoplasmic reticulum, a chromatoid body and some small clusters of dense particles. During spermatid development, the Golgi apparatus is displaced to the distal or postnuclear cytoplasm and adopts a more loosely arranged and more vesicular structure. It appears to diminish in volume by the transfer of vesicles to the surrounding cytoplasm, as described by Clermont (1956). In an attempt

to describe the formation of cytoplasmic droplets in bull and rabbit, Bloom and Nicander (1961) observed the appearance of numerous scattered vesicular and tubular membranous elements in sperm cytoplasm. These authors suggested that these elements are possibly derived from the diminishing Golgi apparatus, the endoplasmic reticulum, which also vesiculates, and mitochondria, which swell and disintegrate near the developing middle piece at this step.

Concomitant with the caudal movement of cytoplasm during spermatid development is the appearance of a system of cytoplasmic filament extending from the nuclear membrane and terminating freely in the cytoplasm just caudal to the acrosome. This system of filaments is microtubular in nature and was termed the manchette by von Lenhossek in 1898 (von Lenhossek, 1898). The involvement of the manchette in reorganization of spermatid cytoplasm has long been speculated (McIntosh and Porter, 1967, Illison, 1968). Fawcett et al (1971) provided evidence that, in a number of species, the manchette may act as a framework or conveyer for transport of vesicles in cytoplasm.

It was also noted that late in spermiogenesis the caudal spermatid cytoplasm is invaginated by processes of Sertoli cell cytoplasm, and it was postulated that these processes were responsible for "pulling off" a large bulk of spermatid cytoplasm (de Krester, 1969, Morales and Clermont, 1982). This large bulk of cytoplasm is termed residual body (corps residual de Regaud) by Regaud (1901). Associated with the invagination of spermatid cytoplasm by Sertoli cell processes, there is a progressive movement of the spermatid toward the lumen of the seminiferous tubule. That portion of the cytoplasm destined to form the residual body is linked to the spermatid by progressively attenuating connections. All connections are lost eventually, and the residual bodies are retained and phagocytosed by the adjacent Sertoli cell (Kingslev-Smith and Lacy, 1959, Lacy, 1960) while the sperm is released to the lumen.

Practically all of the various organelles of the spermatid, including the Golgi complex, endoplasmic reticulum, ribosomes, lipid inclusions, mitochondria, microtubular remnants of the manchette and electron-dense remnants of the chromatid

body along with a great part of cytoplasm, are incorporated into the residual body. The released spermatozoa, however, do retain a small portion of cytoplasm along its mid-piece. This remnant of cytoplasm is the cytoplasmic droplet. The contents of cytoplasmic droplet include some membranous elements termed saccular elements, that aggregate together at one pole of the cytoplasm (Hermo et al., 1988). The most interesting observation is that these saccular elements presumably derived from the spermatid are never found in the residual body during the segregation of spermatid cytoplasm (Lacy 1960, Dietert, 1966, Sapsford et al., 1969, Fouquet, 1974). Conversely, most organelles found within the residual body are not found within the cytoplasmic droplet (Hermo et al., 1988). Such a selective segregation procedure not only suggests that a highly regulated mechanism of cytoplasm reorganization is involved, but also leads one to speculate that the saccular elements retained by the spermatozoa may have a potential biological role.

2) CYTOPLASMIC DROPLETS IN EPIDIDYMAL SPERMATOOZOA

The position of the cytoplasmic droplet relative to the middle piece of the flagellum varies according to the location of the spermatozoon within the lumen of the excurrent duct system. Hermo et al. (1988) observed that in efferent ducts of rats, droplets were arranged around the neck region of spermatozoa flagellum, while in the initial segment and caput epididymidis, the position of the droplets was displaced near the annulus or the point of junction between the middle and principal piece of the flagellum. In the corpus epididymidis, the droplet, still located at the level of the annulus, was noted to be displaced laterally and in the process of pinching off, in the cauda epididymidis, most spermatozoa appeared free of droplets. Similar observations have been reported on different species (Bloom and Nicander, 1961, Bedford, 1975, 1979, Kaplan et al., 1984, Barth and Oko, 1990).

The fate of the cytoplasmic droplets in epididymidis has been studied by several authors. Temple-Smith (1984) identified a region of specialized principal cells in brushtailed possum epididymidis that sequester and phagocytose free intact droplets from the luminal milieu. This process appeared to be selective in that droplets still

attached to spermatozoa were not engulfed. Hermo et al (1988) suggested that when droplets detach from rat spermatozoa, they quickly break up and release their contents directly into the luminal fluid. Using tracer studies with cationic ferritin, various internal structures of the cytoplasmic droplets were seen within the endocytic organelles of the epididymal clear cells, suggesting a possible endocytic role of clear cells in the disposal of cytoplasmic droplets in rats.

3) CHARACTERIZATION OF CYTOPLASMIC DROPLETS

Since its first description in 1909 by Retzius, the cytoplasmic droplet has been studied morphologically by various investigators. Retzius (1909)'s original description of the bull's cytoplasmic droplet containing a dark, oval body was confirmed later by Redenz (1924). Recent morphological studies of cytoplasmic droplets in rats (Herms et al, 1988), using electron microscopy, revealed numerous flattened membranous saccular elements commonly aggregated at one pole. The saccular elements appear to be in close proximity to the plasma membrane delimiting the cytoplasmic droplet, though without any signs of continuity. The asymmetrical distribution of the saccular elements is not observed in several other animal species studied (ram and rabbit by Bloom and Nicander, 1961, Brushtailed possum by Temple-Smith, 1984). Other elements found in rats' cytoplasmic droplets include some spherical vesicles of 60-70 nm diameter in proximity to the saccular elements and some 35 nm diameter and electron-dense particles scattered randomly within the cytoplasm (Herms et al, 1988). The saccular elements, however, are by far the most abundant material within the cytoplasmic droplets in all animal species studied.

The nature of these saccular elements remains enigmatic. Early investigators established the presence of argentophilic and osmiophilic elements in the cytoplasmic droplets from testis and epididymis of various species (Weigl, 1912, Gatenby and Woodger, 1921, Bell, 1929, Gatenby and Collery, 1943, Gresson and Zlotnik, 1945, 1948). This observation was universally interpreted as an indication that the cytoplasmic droplet contained Golgi apparatus and was supported by subsequent studies (Cavazos and Melampy, 1954). Bloom and Nicander (1961) suggested that the

contribution of the endoplasmic reticulum to the content of the cytoplasmic droplet should also be considered. In view of the close relationship between the Golgi apparatus and lysosomes as postulated by Novikoff (1961) and the subsequent confirmation of the role of Golgi complex in the origin of primary lysosomes in a variety of tissues (Novikoff et al., 1964), Dott and Dingle (1968, 1969) suggested a relationship of cytoplasmic droplets and lysosomes. Other investigators suggested that the saccular elements may represent a unique membranous element with little relationship to other organelles (Hermo et al., 1988).

a) Isolation of cytoplasmic droplets

The first attempt to isolate the cytoplasmic droplet was performed by Dott and Dingle (1968) who employed a multi-step centrifugation and filtering method to separate spermatozoa from cytoplasmic droplets in bull semen. The separation of spermatozoa from droplets appeared complete although purity or possible contamination by other semen component in their droplet preparation was not tested. Garbers et al. (1970) criticized traumatic nature of such isolation procedure to the integrity of the cytoplasmic droplets. In an attempt to separate spermatozoa from cytoplasmic droplets, which were shown to contaminate the enzyme profiles of the former, Harrison and White (1969) devised a method using slow cooling and storage in milk diluent overnight to isolate cytoplasmic droplets from semen of bull, boar and ram. Using a simple discontinuous sucrose-density gradient, Garbers et al. (1970) managed to separate cytoplasmic droplet from bovine epididymal spermatozoa, yielding a preparation of droplets that retained the basic morphology of the organelle. In an attempt to study biochemically the differences in various enzyme activities between rat cytoplasmic droplets that are still retained by spermatozoa and those that have detached from spermatozoa (termed "immature" and "mature" droplets, respectively), Roberts et al. (1976) devised a protocol employing repeated tissue homogenization to mechanically force the detachment of "immature" droplets from spermatozoa. The resultant preparation of spermatozoa and droplets were then passed through a column of dry glass wool, which preferentially binds the flagella of

spermatozoa, yielding a preparation of cytoplasmic droplets

b) Polypeptide composition

The polypeptide composition of the membranous saccular elements of cytoplasmic droplets was first analyzed by Kaplan et al (1984) using two-dimensional gel electrophoresis. About 20 polypeptides that are mostly acidic were identified with Coomassie Blue and approximately 100 polypeptides were revealed with silver-staining method. The authors also reported that the polypeptide composition of saccular elements, which was estimated to compose 87.9% of total membrane of cytoplasmic droplets, shows little resemblance to that of the spermatozoa plasma membrane and concluded that the saccular elements are distinct structures from plasma membrane.

c) Enzyme contents

Many investigators have studied the enzymatic contents of the cytoplasmic droplets in various species. Information as such could be helpful in recognizing the potential function of the droplet and also in deducing the origin of the droplet's contents by revealing their similarity in enzyme profile to other organelles. Dott and Dingle (1968) reported that in bull and ram isolated spermatozoa retaining cytoplasmic droplets contained higher activities of several enzymes common in lysosomes, than spermatozoa that were devoid of cytoplasmic droplets. Based on such findings, the authors suggested that the cytoplasmic droplets contain lysosomal bodies. These authors further suggested that the association of infertility and high proportion of ejaculated spermatozoa with retention of the droplets is due to the large quantity of hydrolases within the droplet that interfere with the fertilization process. The lysosomal nature of the cytoplasmic droplets was further supported by Garbers et al (1970) who studied the enzymatic profile of isolated cytoplasmic droplets from bovine.

Cytochemical markers well known to label various compartments of the Golgi apparatus (middle saccules NADPase, trans saccules TPPase, trans tubular network

CMPase) do not show any reaction to the saccular elements or other vesicular elements of the cytoplasmic droplets (Clermont et al , 1981, Tang et al , 1982, Thorne-Tjmosland et al , 1988, Hermo et al , 1988) In addition, G-6-Pase as a marker for endoplasmic reticulum failed to react with rat cytoplasmic droplets (Hermo et al , 1988) These findings, combined with the morphological dissimilarity of the cytoplasmic droplet contents with any other known organelles, led Hermo et al (1988) to speculate that contents of the cytoplasmic droplet is unique, although, as these authors pointed out, the possibility that the saccular elements are derived from organelles in spermatids that had lost their enzymatic characteristics cannot be discounted

In bovine cytoplasmic droplets, Garbers et al (1970) reported activities of several enzymes associated with intermediary metabolism, namely, aldolase, lactate dehydrogenase, sorbitol dehydrogenase and glutamate oxaloacetate transaminase Harrison and White (1972a, b) also noted the activities of three glycolytic enzymes, namely hexokinase (HK), glucose phosphate isomerase (GPI) and lactate dehydrogenase (LD), in bull, boar and ram cytoplasmic droplets Using high speed sedimentation, it was further noted that HK was partly membrane bound while GPI and LDH were entirely soluble in cytoplasmic droplets (Harrison and White, 1972b) Despite the presence of the various metabolic enzymes, incubation of isolated bovine cytoplasmic droplets with 5 mM glucose did not show measurable substrate utilization, oxygen consumption or lactic acid production (Garbers et al , 1970) Similar results was noted using 6 mM succinic acid as substrate (Garbers et al , 1970) These findings led Garbers et al (1970) to conclude that the cytoplasmic droplets are metabolically inert This view, however, was not supported by Roberts et al (1976) who, using [¹⁴C]-glucose as substrate, showed that rat cytoplasmic droplets were capable of synthesizing inositol in spermatozoa Interestingly, such activity was apparently higher in droplets that were still retained in spermatozoa These authors speculated that cytoplasmic droplets is important in spermatozoa maturation in the epididymis possibly through its role in inositol synthesis and metabolism

A difference in enzyme activity in cytoplasmic droplets of spermatozoa isolated from different segments of the epididymis and more distal part of the excurrent ductal system was noted. Roberts et al (1976) compared the profile of activities of ten different enzymes in "immature" rat cytoplasmic droplets (i.e. those that are still retained by epididymal spermatozoa) to the "mature" ones (i.e. those that detached from spermatozoa). Of the enzymes investigated, most of which were of lysosomal origin previously reported in the studies by Dott and Dingle (1968) and Garbers et al (1970), the activities were significantly higher in immature cytoplasmic droplets. Such a differential pattern of enzyme activity was also noted in other mammalian species. Using cytochemical methods at the light microscopic level, Montem and Glover (1972) localized alkaline phosphatase activity in rabbit and ram cytoplasmic droplets. The enzyme activity in rabbit cytoplasmic droplet was found to decrease significantly during spermatozoon epididymal transit. The additional finding of a reduction in alkaline phosphatase activity in degenerating spermatozoa, induced by artificial cryptorchidism, led these authors to speculate a potential role of the enzyme in early maturation of spermatozoa in the epididymis.

Despite the efforts of the aforementioned investigators on the study of cytoplasmic droplets, there remains several crucial questions to be answered. First, what is the function of these saccular elements? The saccular elements not only are the most prominent structures within cytoplasmic droplets of all animal species studied, but they are also selectively retained within the cytoplasmic droplet during late steps of spermiogenesis. These findings infer that these saccular elements might have a specific role, which may be the key to understand the function of cytoplasmic droplets. Secondly, different studies have clearly indicated a regional variation in activities of different enzymes in cytoplasmic droplet from different segment of the excurrent ductal system. A crucial question that arises is why such a variation evolved. Finally, are these saccular elements remnants of organelles in late spermatids, and, if so, what are they? Although lysosomal enzymes activities have been found in cytoplasmic droplets, direct evidence on whether these enzymes are associated with the saccular elements is still lacking. Knowing the origin of these

elements may be helpful in unraveling the potential functional role of such structures

MATERIALS AND METHODS

MATERIALS AND METHODS

I ULTRASTRUCTURAL ANALYSIS OF LATE SPERMATID IN THE TESTIS

Testes were obtained from adult Sprague-Dawley rats weighing from 350 - 450 gm. Each rat was anesthetized via an intraperitoneal injection of sodium pentobarbital. The testes of the animals were first perfused with lactated Ringer's solution (Abbott laboratories Ltd., Montreal, PQ) followed by 2.5 % glutaraldehyde (M.F.C.A. Ltd., Montreal, PQ) in 0.1 M sodium cacodylate (M.E.C.A. Ltd., Montreal, PQ) buffer containing 0.5 % CaCl_2 (Sigma Chemical Co., St. Louis, MO) using a retrograde abdominal aorta perfusion technique. The abdominal cavity was opened and the abdominal aorta was carefully exposed and cleared of connective and adipose tissues. A ligature was placed around the abdominal aorta just superior to the left renal artery and left untied. Testes were then carefully removed from the scrotum. Circulation in the lowest part of the aorta was clamped off with a hemostat and a 18-gauge needle (Becton Dickinson & Co., Rutherford, N.J.) was inserted in a retrograde direction into the abdominal aorta superior and proximal to its bifurcation. The ligature was tied as soon as the flow of lactated Ringer's solution was established and the left renal vein was cut open to allow exit of fluid. The flow of Ringer's was ceased as soon as the testis was observed to be cleared of blood. After trimming off the connective and adipose tissue, the testes were sliced into small pieces (0.5 mm³) and placed in the same fixation for 2 hr at 4°C. The postfixation, dehydration, Epon-embedding, sectioning and staining procedures were identical to those described below in section V.1. After postfixation with 1% aqueous osmium tetroxide, some of the tissue samples were treated with 1% tannic acid (Sigma chemical Co., St. Louis, MO) in cacodylate buffer (pH 7.0) (3 x 20 min) and finally washed in 1% sodium sulfate in cacodylate buffer (20 min) (Simionescu and Simionescu, 1976) before Epon-embedding.

Electron micrographs were taken with a Philips 400 electron microscope. Stages of spermiogenesis were determined using criteria described by Leblond and Clermont (1952b).

II STEPWISE PROCEDURES FOR ISOLATION AND SUBI RACTIONATION OF CYTOPLASMIC DROPLETS

1) PERFUSION AND DISSECTION OF EPIDIDYIMIDIS

Spermatozoa were obtained from the epididymidis of adult Sprague-Dawley rats weighting from 350 - 450 gm. Nine rats were used per experiment and they were anesthetized with 0.4 ml sodium pentobarbital. The epididymidis of the animals were perfused with lactated Ringer's solution using a retrograde abdominal aorta perfusion technique as described above in section I. After trimming off the connective and adipose tissue, the epididymides were separated according to Reid and Cleland (1957) into the caput (including the initial segment, the proximal and distal caput) and cauda (including the proximal and distal cauda). All the subsequent procedures were performed separately for the caput and cauda epididymidis on ice or at 4°C. The tissues were rinsed in a buffer containing 0.25 M sucrose (Sigma Chemical Co., St. Louis, MO), 50 mM Tris-HCl (Sigma Chemical Co., St. Louis, MO) and 25 mM KCl (Sigma Chemical Co., St. Louis, MO) at pH 7.4 (STK buffer). Protease inhibitors including phenylmethyl-sulfonyl fluoride at 1.0 mM (Sigma Chemical Co., St. Louis, MO) and aprotinin at 100 units/ml (Sigma Chemical Co., St. Louis, MO) were added to this buffer. For the galactosyltransferase biochemical assay with trypsinization (section VI 1), this last step of protease inhibitor addition was omitted.

2) ISOLATION OF EPIDIDYMAL SPERMATOOZA AND CYTOPLASMIC DROPLETS

The caput and caudal portions of the epididymidis were separately sliced into 2 mm sections in STK buffer. The minced tissue suspension was then vortexed lightly to facilitate the exudation of spermatozoa from the lumen of the epididymidis. This suspension was then filtered through a 150 µm Nitex netting (Thompson, Montreal, PQ) to remove epithelial tissue and to obtain a spermatozoa suspension in STK buffer. The spermatozoa suspension was washed by a 15-min, 3000 g centrifugation. The sperm pellet was resuspended in 5.0 ml of STK buffer and the supernatant was centrifuged at 80,000 g for 15 min to collect any freed cytoplasmic droplets into a pellet which was then resuspended in 1.0 ml of STK buffer. The pellet suspensions

were pooled and passed through a 1.5 inch-long 20-gauge needle (Becton Dickinson & Co., Rutherford, NJ) four times using a 10 cc syringe (Becton Dickinson & Co., Rutherford, NJ), which, as evaluated by phase-contrast microscopy, detached the cytoplasmic droplets still retained on the sperm tails

3) SUBFRACTIONATION OF THE CYTOPLASMIC DROPLETS

The resulting homogenate was centrifuged at 150 x g for 10 min in order to float the detached cytoplasmic droplets into the supernatant. The supernatant (S fraction) was loaded onto a 4-step sucrose discontinuous gradient (0.6 M/0.8 M/1.0 M/1.2 M) prepared in 50 mM Tris-HCl and 25 mM KCl buffer, pH 7.4 and centrifuged at 200,000 x g for 1.5 hr using a Beckman SW 41-T1 rotor. Each of the cellular fractions collected from the 5 interfaces of the gradient together with the originating S fraction and pellet (P fraction) were analyzed by electron microscopy and assayed for protein concentration and galactosyltransferase, sialyltransferase and acid phosphatase enzyme activities.

III PREPARATION OF IMMUNE SERUM AGAINST THE SACCULAR ELEMENT FRACTION

Fraction 3 (0.8 M/1.0 M STK) from the discontinuous gradient prepared from whole epididymidis was collected and solubilized in 0.5 M Tris-HCl (pH 6.8) containing 2.0% SDS and 5.0% β -mercaptoethanol for 5 min at 100°C. The denatured proteins were then emulsified in an equal volume of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO) and 100 μ l of this suspension (1 μ g protein/ μ l) was injected into each popliteal lymph node (Newbould, 1965) of a New Zealand white rabbit. The rabbits were boosted intramuscularly with the antigens emulsified in Freund's incomplete adjuvant (Gibco Laboratories, Grand Island, NY) at 3-wk intervals and bled 7-10 days after each boost. The immune serum was stored frozen at -70°C.

IV ANALYSIS OF POLYPEPTIDE COMPOSITIONS

1) SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Cellular fractions collected from each interface of the discontinuous gradient and from the supernatant and pellet of the first centrifugation were diluted in 50 mM Tris-HCl and 25 mM KCl buffer, pH 7.4 (TK) and pelleted. Each pellet was resuspended in TK buffer and assayed for total protein concentration as described in section VI.3. Samples containing 30 to 60 µg of protein from each cellular fraction were solubilized in a solution of 2% sodium dodecyl sulfate (SDS) (Mallinckrodt Canada Inc., Pointe-Claire, PQ) and 5% β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) for 5 min at 100°C and loaded on a linear gradient (8-18%) SDS-discontinuous polyacrylamide gels (SDS-PAGE) according to the procedure described by Laemmli (1970).

Apparent molecular masses were determined from the mobility of the following standard proteins (Pharmacia low molecular weight proteins, Piscataway, NJ): phosphorylase b (MW=94 K), albumin (MW=67 K), ovalbumin (MW=43 K), carbonic anhydrase (MW=30 K), Soybean trypsin inhibitor (MW=20.1 K) and α-lactalbumin (MW=14.4 K). The gels were stained with Coomassie Brilliant Blue (Bio-rad, Richmond, CA).

2) WESTERN BLOTTING

Electrophoretic transfer of polypeptide bands, from the gels of fraction 3 isolated from caput, cauda epididymidis or whole epididymis, to immobilon paper (Millipore, Mississauga, ON) was carried out on a Hoefer Transpher Apparatus (Hoefer Scientific Instruments, San Francisco, CA) according to the techniques of Towbin et al. (1979) and Towbin and Gordon (1984). After SDS-PAGE, preparative gels of polypeptides were immediately transferred onto methanol-pretreated immobilon paper in a solution of 25 mM Tris-HCl, 192 mM glycine and 20% methanol (pH 8.3). Electrophoretic transfer was carried out at 0.5-0.6 A for 3-5 hrs. After removal from the transfer

apparatus, the blots were washed in three 15-min changes of 10 mM Tris-HCl, 0.9% NaCl and 0.5% Tween-20 (pH 7.4) at room temperature. For a permanent record of the protein banding pattern, the immunoblot blots were stained with Coomassie Brilliant Blue.

3) IMMUNOBLOTTING

Washed immunoblot strips transferred with polypeptide bands of the saccular elements of the cytoplasmic droplet were first blocked with 5% goat serum in TWBS (20 mM Tris-HCl buffer with 0.9% saline and 0.1% Tween-20) for 2 hr at room temperature. The strips were then incubated for 2 hr at room temperature with 1) affinity-purified antibodies against α 2,6 sialyltransferase (see section VIII), or 2) antiserum against β 1,4 galactosyltransferase (see section VIII), or 3) polyclonal antibodies raised in rabbits against the saccular elements of the cytoplasmic droplet. Antibodies were diluted in TWBS with 0.1% bovine serum albumin (BSA) and 1% goat serum. The dilutions used were determined empirically and reflect the optimum concentration and labeling that minimized non-specific labeling. The strips were subjected to four 5-min rinses in TWBS, followed by a 30-min wash with 5% goat serum in TWBS. After incubation for 2 hr at 37°C in a 1/1000 dilution of secondary antibody (alkaline phosphatase-conjugated F(ab)₂ goat anti-rabbit immunoglobulin G [IgG], Cappel-Cooper Biomedical Inc., Malvern, PA), the strips were again subjected to four 5-min rinses in TWBS, and then rinsed for 2 min in 50 mM Na-glycinate (Sigma Chemical Co., St. Louis, MO) with 0.5% Tween-20 (pH 9.6). The phosphatase reaction was developed according to McGadey (1970) by incubating blots in a solution composed of 0.01% of Nitro blue tetrazolium (Sigma Chemical Co., St. Louis, MO), 0.005% and 5-bromo-4-chloro-3-indolyl phosphate and 4 mM of MgCl₂ of in the 50 mM Na-glycinate solution for 10-20 min. Controls consisted of replacing primary antibodies with affinity-purified antibodies against isoactins (Oko et al., 1991) or against α -tubulins (Hermo et al., 1991a) in seminiferous epithelial cells. In the case of the polyclonal antibodies raised, preimmune serum was used as a control.

V. PREPARATION OF THE CELLULAR FRACTIONS FOR ELECTRON MICROSCOPY

1) EPON EMBEDDED SECTIONS

Cellular fractions collected from each interface of the discontinuous gradient were diluted in STK buffer and pelleted. Pellets were fixed with 2.5 % glutaraldehyde buffered in 0.1 M sodium cacodylate containing 0.05 % CaCl_2 , pH 7.4, overnight at 4°C. Fixed pellets were then submersed in 2% type IX agarose (Sigma Chemical Co., St. Louis, MO), allowed to solidify, and washed twice with the 0.1 M cacodylate buffer. The pellets were subsequently post-fixed with 1.5% potassium ferrocyanide (Fisher Scientific Co., Fair Lawn, NJ) reduced 10% osmium tetroxide (M.E.C.A. Ltd., Montreal, PQ) (Karnovsky, 1971) for 1 hr at 4°C. After being washed twice in 0.1 M cacodylate buffer, the pellets were dehydrated in a graded series of ethanol solutions 50%, 60%, 70%, 90%, 100% (3 times), each for 10 min at room temperature. The pellets were then passed through two 15-min washes of propylene oxide (Anachemia Canada Inc., Montreal, PQ), infiltrated in a 1:1 mixture of propylene oxide/Epon 812 (M.E.C.A. Ltd., Montreal, PQ) for 1 hr, a 1:2 mixture for 6 hr, and a 1:3 mixture for 15 hr at room temperature on a rotator at constant agitation. Tissues were then placed in pure Epon 812 for 6 hr within a vacuum, embedded in block-molds and incubated at 60°C for 48 hr. Thin tissue sections of silver interference colors were cut on a Reichart-Jung ultramicrotome with a diamond knife, mounted on 300 mesh uncoated copper grids and stained with 4 % uranyl acetate (M.E.C.A. Ltd., Montreal, PQ) for 5 min and 6% lead citrate (M.E.C.A. Ltd., Montreal, PQ) for 2 min (Stemmpak and Ward, 1964, Reynold, 1963). The ultrathin sections were examined under a Philips 400 electron microscope.

2) ULTRATHIN FROZEN SECTIONS

Cellular fractions were fixed with 4% paraformaldehyde (Fisher Scientific Co., Fair Lawn, NJ) in 0.1 M sodium cacodylate buffer (pH 7.4) with 0.1% CaCl_2 (w/v) for 1 hr at 4°C. The fixed suspensions were then pelleted at 10,000 x g and the resulting pellets were embedded in 2% type IX agarose, allowed to solidify, washed

twice with 0.1 M cacodylate buffer containing 7% sucrose and equilibrated for a minimum of 30 min with 2.3 M sucrose (Tokuyasu, 1978). The pellets were frozen directly in liquid-nitrogen-cooled propane and stored in liquid nitrogen (Linde Union Carbide, Toronto, ON). All the subsequent steps were based on the frozen section procedures developed by Tokuyasu (1978, 1980), Griffiths et al (1984), and Geuze et al (1984). A Reichart-Jung ultramicrotome equipped with an FC-4D cryochamber equilibrated at -100°C was used for tissue sectioning. Copper grids (200 mesh) having formvar films were carbon-coated and then rendered hydrophilic using a glow-discharge apparatus. Ultrathin sections were cut, picked up with a copper loop filled with a 2.3 M sucrose solution and transferred onto the grids. Each grid was then kept at room temperature on a drop of 5% fetal calf serum (Gibco Laboratories, Grand Island, NY) in 20 mM Tris-HCl buffer, 0.9% saline buffer at pH 7.4 (FBS) containing 0.1% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, MO).

The staining and embedding procedure of Griffiths et al (1984), originally described by Tokuyasu (1978), were used. The grids containing the sections were first rinsed for 10 min in doubly-deionized water (ddH₂O) and then floated for 5 min on drops of 2% aqueous uranyl acetate which had been adjusted to pH 7.3 with oxalic acid (Sigma Chemical Co., St. Louis, MO) and ammonium hydroxide (Sigma Chemical Co., St. Louis, MO). After rinsing twice for 1.5 min on drops of ddH₂O and transferred onto drops of 0.5% methyl cellulose (Lab. Grade Ac5900, Anachemia Canada Inc, Montreal, PQ) containing 0.4% aqueous uranyl acetate for 10 min, the grids were picked up with a gold loop blotted to remove excess methyl cellulose, dried and examined on a Philips 400 electron microscope.

VI ENZYME ASSAYS OF CELLULAR FRACTIONS

1) GALACTOSYLTRANSFERASE ASSAY

Galactosyltransferase (UDP-Gal 2-acetamido-2-deoxy-D- glucosyl-glycopeptide galactosyltransferase) activity in the cellular fractions obtained from the discontinuous gradient were assayed using a modified technique for the Golgi apparatus (Bretz and Staubli, 1977, Howell et al., 1978) based upon the transfer of UDP-[³H]galactose to

the exogenous acceptor, ovomucoid. The donor sugar consisted of UDP-[³H] galactose (Uridine diphosphate galactose, [galactose-1-³H(N)]-, Du Pont Inc., Markham, ON) that was dried with nitrogen and resuspended with UDP-galactose (Uridine-5' Diphosphogalactose, sodium salt, Sigma Chemical Co., St Louis, MO) to a specific activity of 0.045 uCi/nmol. The incubation mixture contained 0.0441 mM of UDP-galactose (0.2 uCi), 30 mM sodium cacodylate buffer (pH 6.5), 2.0 mM adenosine triphosphate (disodium salt) (Sigma Chemical Co., St Louis, MO), 30 uM MnCl₂ (Sigma Chemical Co., St Louis, MO), 30 mM β-mercaptoethanol (Sigma Chemical Co., St Louis, MO), 0.2 % (v/v) Triton X-100 (Sigma Chemical Co., St Louis, MO), 4.0 mg/ml ovomucoid (Sigma Chemical Co., St Louis, MO) and 30 ul of the cell fraction sample (5-20 ug protein) giving a final volume to the reaction mixture of 100 ul. Incubations were carried out for 15 min at 37°C and stopped by the addition of 1.0 ml of ice-cold 1% phosphotungstic acid (JT Baker Inc., Phillipsburg, NJ) in 0.5 N HCl. The mixtures were kept on ice for 30 min to allow precipitation of the protein and then pelleted by centrifugation. The pellets were resuspended and repelleted twice in 1.0 % phosphotungstic acid. After being dried overnight, the pellets were dissolved in 0.5 ml of 1.0 N sodium hydroxide (Sigma Chemical Co., St Louis, MO) and incubated at 65°C for 1 hr. 0.1 ml of each sample was mixed with 5.0 ml of scintillation cocktail (Opti-flour, Packard Instrument Co., Downer Grove, Ill). The radioactive content of the pellets was determined in a Packard Spectrometer model 460 (Packard Instrument Co., Downer Grove, Ill) and cpm converted to dpm by the channels-ratio and external standard methods (Wang and Willis, 1965).

The effect of the protease trypsin on galactosyltransferase activity was studied as follows. 70 ul (20-30 ug protein) of cell fraction from fraction 3 (interface between 0.8 M and 1.0 M sucrose buffer) of caput epididymal spermatozoa was incubated for 1 hr at 0°C with trypsin (type IX from porcine pancreas, Sigma Chemical Co., St Louis, MO) at different concentrations (0.1 ug/ml, 1 ug/ml, 10 ug/ml, 50 ug/ml and 100 ug/ml) in 50 mM Hepes buffer (Sigma Chemical Co., St Louis, MO) with 5 mM KCL, 1mM CaCl₂ and 1 mM MgCl₂, in the presence or absence of 0.1% (v/v) Triton

X-100 The galactosyltransferase assay was performed as described above. The enzyme activity of each trypsinized sample was expressed as a percentage of the total enzyme activity of a control sample without trypsinization.

2) SIALYLTRANSFERASE ASSAY

The assay for sialyltransferase (CMP-N-acetyl-neuraminyl-4-galactosyl-glycoprotein N-acetylneuraminyl-transferase), was modified from Bretz et al (1980). The donor sugar consisted of CMP-[³H] N-acetyl-neuraminic acid (NANA) (Cytidine 5'-monophosphate sialic acid, [sialic-9-³H]-, Du Pont Inc., Markham, ON) that was dried and resuspended with CMP-NANA (Cytidine 5'-monophospho-(NANA), ammonium salt, Sigma Chemical Co., St. Louis, MO) to a specific activity of 4.3 uCi/umol. The incubation mixture contained 1.0 mM of CMP-NANA (0.43 uCi), 100 mM cacodylate buffer (pH 5.8), 40 mM β-mercaptoethanol, 0.4% (v/v) Triton X-100, 17.5 mg/ml of asialofetuin (Sigma Chemical Co., St. Louis, MO) as an exogenous acceptor for sialyltransferase and 60 ul of the cell fraction sample (10-40 ug protein) giving a final volume of 100 ul. Incubations were carried out for 30 min under identical conditions as in the galactosyltransferase assay above. The washing and counting procedures were also identical to those used in the galactosyltransferase assay.

3) ACID PHOSPHATASE ASSAY

Acid phosphatase activity was determined at 37°C by incubating 50 ul (10-30 ug protein) of each cell fraction from the discontinuous sucrose gradient with 2.0 mM para-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in 100 mM sodium acetate (Sigma Chemical Co., St. Louis, MO) buffer (pH 5.0) in a total volume of 500 ul for 15 min. The reaction was stopped by adding 1.0 ml of 0.25 M NaOH. The released para-nitrophenol was measured spectrophotometrically (Beckman Du-8 spectrophotometer) at 405 nm using a para-nitrophenol standard curve.

The amount of protein in the cell fractions for all of the enzyme assays was estimated as described by Bradford (1976) (Bio-Rad Richmond, CA), using bovine serum albumin as the standard.

VII ENDOGENOUS GLYCOSYLATION ASSAYS

Isolated saccular elements from fraction 3 (0.8 M/1.0 M STK) of the discontinuous gradient, prepared from caput epididymidis, were assayed for endogenous glycosylation as described previously for rat liver Golgi fractions (Bergeron et al., 1982, 1985). For galactosylation, the reaction mixture, containing 2.5 μ Ci of UDP-[³H] galactose, 30 mM sodium cacodylate buffer at pH 6.5, 2.0 mM adenosine triphosphate (disodium salt), 30 mM MnCl₂, 30 mM β -mercaptoethanol, 35 μ g protein of the sample fraction and protease inhibitors (1.0 mM PEFABLOC (Centerchem, Inc., Stamford, CT), 200 units/ml aprotinin, 1.0 μ M papstatin (Sigma Chemical Co., St. Louis, MO)), was incubated at 37°C for 15 min. For sialylation, the reaction mixture, containing 2.5 μ Ci CMP-[³H] NANA, 100 mM sodium cacodylate buffer at pH 5.8, 40 mM β -mercaptoethanol, 35 μ g sample protein and protease inhibitors (as above), was incubated at 37°C for 15 min. The glycosylation reactions were stopped by adding equal volume of 10% trichloroacetic acid (Fisher Scientific Co., Fair Lawn, NJ). The precipitated proteins were dissolved in sample preparation buffer containing 4.0 M urea and separated on a 5-15% SDS-PAGE, as described in section IV.1. After staining, the gels were impregnated with ENHANCE (Du Pont Inc., Boston, MA) and processed for radioautography (Eastman Kodak Co., X-OMAT AR, Rochester, NY).

VIII IMMUNOCYTOCHEMISTRY OF CELLULAR FRACTIONS AND EPIDIDYMAL TISSUE

The source of the anti- β 1,4 galactosyltransferase was from Dr. E. Berger (Physiologisches Institut, Zurich, Switzerland) and was antiserum N₇ raised to native human milk galactosyltransferase (Childs et al., 1986). The anti- α 2,6 sialyltransferase was from Dr. J. Paulson (Cytel Corp., San Diego, CA) and was IgG affinity purified from bacterially expressed sialyltransferase (Taatjes et al., 1988). The anti-TGN38 was antiserum obtained from Dr. K. Howell (University of Colorado, Denver, CO) and has been described and characterized in Luzio et al. (1990). Antibodies against the saccular element (anti-SE) was prepared as described in section III. These antibodies were assayed at both high and low concentrations on the tissue

The dilutions used therefore reflect the optimum concentration and labeling for each antibody preparation that minimized non-specific labeling

1) LIGHT MICROSCOPY

Rats used for the preparation of light microscopy were fixed via perfusion through the abdominal aorta, first with lactated Ringer's solution followed by Bouin's fixative for 10 min. The entire procedure was carried out at room temperature. After the testes and epididymidis were removed, they were cut in half longitudinally, and placed in the same fixative overnight. On the following day, the tissue was subjected to several washes in 70% ethanol. The testicular and epididymal tissue was then subjected to routine histological procedures and embedded in paraffin.

Paraffin sections (5 μ m in thickness) were cut and mounted onto glass slides. The tissue sections were deparaffinized with xylene (Fischer Scientific Co., Fair Lawn, NJ) and hydrated in graded ethanol solutions. During hydration the sections were incubated in 70% ethanol containing 1% lithium carbonate (Sigma Chemical Co., St. Louis, MO) for 5 min in order to inactivate any residual picric acid remaining from the fixation procedure. In order to eliminate endogenous peroxidase activity, the sections were also incubated for 5 min in ethanol containing 1% (v/v) hydrogen peroxide (Sigma Chemical Co., St. Louis, MO). Following hydration, the sections were washed first in a 300 mM glycine solution (Sigma Chemical Co., St. Louis, MO), to block any free aldehyde groups, and then in distilled water. The tissue sections were placed briefly in 20 mM Tris-HCl buffer with 0.9% saline at pH 7.4 (TBS) containing 0.1% (w/v) bovine serum albumin.

The sections were subsequently blocked in TBS containing 10% goat serum (Sigma Chemical Co., St. Louis, MO) for 15 min followed by incubation for 1 hr with primary antibodies at various dilutions in TBS. After the completion of the primary antibody incubation, the tissue sections were washed four times, 5 min each, with TBS containing 0.1% Tween-20 (Sigma chemical Co., St. Louis, MO), blocked with 10% goat serum in TBS for 15 min and incubated for 1 hr with goat anti-rabbit IgG conjugated to peroxidase (Sigma Chemical Co., St. Louis, MO) that was diluted

1/250 in TBS

Following several washes in TBS containing 0.1% Tween-20, the immunoperoxidase reaction was developed by incubating the sections for 10 min in a solution composed of 0.03% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in TBS containing 0.01 M imidazole (Sigma Chemical Co., St. Louis, MO), pH 7.6 (Straus, 1982). This permitted the deposition of an insoluble brown polymer at the site of the antigen-antibody reaction (Heyderman, 1979). The slides were subsequently rinsed twice in TBS containing 0.1% Tween-20 (5 min each) followed by two rinses in distilled water (1 min each). The tissue sections were then counterstained with 0.1% methylene blue (Aldrich Chemical Co., Milwaukee, Wis), dehydrated in an ethanol gradient, and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ).

Rabbit preimmune serum at a dilution of 1/100 was used as a control for the polyclonal anti-SE antibodies. Affinity purified antibodies against a host of other membranous proteins, including the insulin receptor and epidermal growth factor receptor, were used as controls for anti-galactosyltransferase, sialyltransferase and TGN-38 antibodies.

2) ELECTRON MICROSCOPY

a) Lowicryl embedded sections

Rats used for electron microscopic immunocytochemistry were perfused with lactated Ringer's solution followed by perfusion for 10 min through the abdominal aorta with 0.25% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer containing 50 mM lysine at pH 7.4. Following perfusion, the epididymides and testes were removed, sliced into small pieces (0.5 mm³), and placed in the same fixation for 2 hr at 4°C. They were subsequently washed several times in 0.15 M phosphate buffer and 0.9% saline (PBS) (pH 7.4) containing 4% sucrose followed by incubation in PBS containing 4% sucrose and 50 mM NH₄Cl for 1 hr at 4°C. The tissue samples were then washed in PBS and dehydrated in a graded series of methanol solution: 30% at 0°C, 50% at -10°C, 70% at -35°C and 90% at -35°C.

(twice) for 15 min each. The samples were subsequently infiltrated with a 1:1 ratio of Lowicryl/90% methanol solution for 1 hr at -35°C and a 2:1 ratio overnight at -35°C. The next day the samples were embedded in Lowicryl K4M (JB EM services Inc., Dorval, PQ) and polymerized at -35°C under UV radiation (Grant et al., 1985). Ultrathin sections were mounted on formvar coated nickel grids.

For immunolabeling, the ultrathin tissue sections on grids were floated for 30 min on drops of 20 mM Tris-HCl buffer with 0.9% saline at pH 7.4 (TBS) containing 0.1% bovine serum albumin and 5% fetal calf serum prior to a 1 hr incubation with primary antibodies. Primary antibodies were diluted to their optimal concentrations with TBS. Following primary antibody incubation, the sections were washed for 5 min (x 4) in TBS containing 0.1% Tween-20, blocked with 5% fetal calf serum in TBS for 15 min, and incubated for 45 min with a 1/20 dilution of goat anti-rabbit IgG conjugated to 8 nm colloidal gold particles (Zollinger Inc., Montreal, PQ) in TBS. The sections were then washed for 5 min in TBS containing 0.1% Tween-20 (x 3) followed by two 5-min washes in distilled water. The control antibodies were identical to those already mentioned under light microscopic immunocytochemistry in section VIII.1. Counterstaining of the tissue sections was done with 4% uranyl acetate (2 min) followed by 6% lead citrate (30 seconds).

b) Ultrathin frozen sections

Preparation of the cellular fractions for frozen sectioning was described earlier (section V.2). All the subsequent steps were performed at room temperature and on the same day. Incubations and washings were done according to Janssen Life Science Products (1985). For immunolabeling, the grids were first incubated for 15 min with a blocking reagent (10% fetal calf serum in TBS (pH 7.4) containing 0.1% bovine serum), followed by incubation with the primary antibodies at their optimal dilution. After three 5-min washes with 1% fetal calf serum in TBS, the grids were again incubated with the blocking reagent for 15 min. The grids were then incubated on drops of colloidal gold (8 nm)-conjugated goat anti-rabbit IgG (Zollinger Inc., Montreal, PQ) at a dilution of 1/20 followed by three 5-min washes in TBS and two 5-min washes in ddH₂O. The staining and methyl cellulose embedding procedures are

already described in section V 2 Controls used were identical to those already mentioned in section VIII 1

IX LECTIN BINDING ANALYSIS OF EPIDIDYMAL SPERMATOZOA

The presence of D-galactose and N-acetyl-D-galactosamine- containing glyco-conjugates were detected by using Ricinus Communis Agglutinin I (RCAI) and Helix Pomatia lectin (HPL), respectively, conjugated to colloidal gold The colloidal gold particles (15 nm) was prepared using the sodium citrate method described by Frens (1973) Direct RCAI-colloidal gold (GC) and direct HPL-colloidal gold (GC) complexes were prepared according to the method of Roth and Binder (1978)

Direct lectin-colloidal gold (15 nm) labeling of ultrathin Lowicryl sections was performed using a 1:5 dilution of lectin- gold complex in 0.15 M phosphate buffer and 0.9% saline (PBS) at pH 7.4 Tissue sections were first incubated at room temperature on a drop of PBS for 5 min and then transferred onto a drop of RCAI-CG or HPL-CG for 1 hr The sections were then washed with PBS followed by double distilled water and dried on filter paper (Whatman International Ltd, Maidstone, England) As controls for RCAI and HPL binding, sections were incubated at room temperature in the presence of blocking sugars D-galactose (0.2 M, Sigma Chemical Co., St. Louis, MO) or N-acetyl-D-galactosamine (0.2 M, Sigma Chemical Co., St. Louis, MO) The tissue sections were counterstained with uranyl acetate and lead citrate, as described in section VIII 2a, and were examined on a Philips 400 electron microscope

X STATISTICAL ANALYSIS

The results of enzyme specific activities of each subfractions in exogenous radioactive biochemical assays were analyzed by single factor analysis of variance test (ANOVA) and Student Newman Keuls (SNK) test to detect any significant difference in enzyme activities localized in each fractions Two-tailed paired Student's t-tests were used to detect any significant difference between the enzyme specific activities of the corresponding subfractions isolated from caput and cauda epididymal spermatozoa

RESULTS

RESULTS

I ULTRASTRUCTURE OF THE CYTOPLASMIC DROPLETS IN THE EXCURRENT DUCT SYSTEM

In the lumen of the efferent ducts and initial segment of the epididymis, the cytoplasmic droplet retains its position as a small outpouching of cytoplasm evenly arranged around the sperm's tail at the level of the neck piece (Bloom and Nicander, 1961, Fawcett and Ito, 1965, Fawcett and Phillips, 1969, Phillips, 1975, Bedford, 1979, Kaplan et al, 1984, Robaire and Hermo, 1988, Hermo et al, 1988)

The ultrastructural features of the contents of cytoplasmic droplets of spermatozoa found in the lumen of the initial segment and caput epididymidis are illustrated in figures 2-4. The major structural feature of the droplet was the presence of numerous flattened saccular elements which tended to be aggregated together and occupy one pole of the droplet (Fig 2). The latter finding was consistent with the observations by phase contrast microscopy on living spermatozoa from caput epididymidis where the saccular elements of the droplet continually encircle, as an aggregate, the outer circumference of the droplet in close proximity to the plasma membrane. The saccular elements were straight or C-shaped in appearance (Figs 2-4). In appropriate sections of the saccular elements it was apparent that they were disc or plate-like structures and not tubules (Figs 2,3). This latter finding was confirmed on grazing sections by electron microscopy in previous studies (Fig 4).

The saccular elements often showed wide areas of close approximation to one another but equally large areas of separation. In other cases, their edges were in close proximity to those of others (Figs 2,3). Areas where saccular elements came in close proximity to one another revealed the presence of an intervening, finely filamentous dense material (Fig 3). In addition, consistent with a previous morphological study of the cytoplasmic droplet (Hermo et al, 1988), a few of the saccular elements appeared to be closely associated with the plasma membrane, although continuity between the two was not observed (Fig 4).

II ULTRASTRUCTURAL ANALYSIS OF LATE SPERMATIDS IN THE TESTIS

The Golgi apparatus undergoes marked changes in its overall configuration and appearance during the course of spermiogenesis (steps 1-19). In early spermatids (steps 1-7) the Golgi apparatus was hemispherical and occupied the cranial pole of the nucleus (Fig 6), where a role in the formation of the acrosome has been demonstrated (reviewed by Clermont et al, 1990). At step 8, the Golgi apparatus was observed at the caudal pole of the nucleus next to the developing tail with a spherical morphology and thereafter was observed free in the cytoplasmic lobe of the spermatid. Morphological analysis of steps 9-15 spermatids revealed a classically oriented Golgi apparatus with stacked saccules and associated vesicles (Fig 7).

By step 16, the Golgi apparatus appeared less intact with dispersed saccules now evident (Fig 8). A notable feature at this step was that several flattened saccular elements were seen distant to the Golgi apparatus. At steps 17 & 18 of spermiogenesis, the Golgi apparatus was no longer observed as a morphologically recognizable structure. This event coincided with the presence of many flattened saccular elements which were observed either freely distributed in the surrounding cytoplasm (Fig 9) or in loose aggregates (Fig 9 inset, Fig 10). It should be noted that some saccular elements also presented a C-shaped appearance which, when cut in a cross section, appeared as circular elements surrounded by a double membrane (Fig 10).

At early step 19 of spermiogenesis some vestiges of the Golgi apparatus are identified as small aggregates of several flattened saccular elements which show no definable orientation and few vesicular profiles (Fig 11). The most salient feature of this step is that numerous saccular elements are loosely distributed throughout the cytoplasm.

Morphological analysis of late step 19 spermatids revealed a segregation of unused mitochondria, granulated bodies, endoplasmic reticulum, ribosomes and other organelles into the residual body destined for phagocytosis by Sertoli cells (Morales et al, 1985). The flattened saccules, on the other hand, congregated within the small mass of leftover cytoplasm in the neck region of the tail which by late step 19 was

recognizable as the cytoplasmic droplet (Fig 12) It should be emphasized that the saccular elements were not found in residual bodies and that the superfluous organelles present in residual bodies were not found in the droplets Furthermore the flattened saccular elements of the droplet were similar in morphological appearance and dimension to those found loosely distributed in the cytoplasm of spermatids beginning at step 16 (compare Figs 4 & 8), and to the flattened saccular elements composing the Golgi seen in earlier steps of spermiogenesis (compare Figs 2 & 7)

III ISOLATION OF SACCULAR ELEMENTS OF THE CYTOPLASMIC DROPLET

Saccular membranous elements of the cytoplasmic droplet were isolated from spermatozoa obtained from both caput (including initial segment, proximal and distal caput) and cauda (including proximal and distal cauda) epididymidis Since the corresponding fractions obtained from spermatozoa of these two regions of the epididymis were morphologically identical, the following description of the isolation procedure and the results obtained were applicable to spermatozoa isolated from both caput and cauda epididymidis To assure that the cytoplasmic droplets were detached from the spermatozoa, the initial sperm suspension was subjected to a shear force by passing it through a 20 gauge needle several times The suspension was then centrifuged at low speed into a supernatant (S) fraction, containing predominantly cytoplasmic droplets, as determined by electron microscopy (Fig 13), and a pellet (P) fraction containing spermatozoa (Fig 14) It is important to note, however, that a substantial number of cytoplasmic droplets were still found in the pellet and thus the supernatant was only representative of a fraction enriched in cytoplasmic droplets

The cytoplasmic droplet-enriched supernatant was subsequently used to subfractionate the droplet on a 4-step sucrose discontinuous gradient By ultrastructural analysis of the 5 resulting fractions, it was found that the saccular elements in question resided mainly in fractions 3 and 4 of the gradient

Fraction 3 of the gradient, corresponding to the interface between 0.8 M and 1.0 M sucrose buffer (sp gravity 1.105/1.130), was comprised of numerous plasma membrane-enclosed droplets containing flattened, straight and C-shaped saccular

elements, resembling those found within droplets in situ except for a loss of cytoplasm (Fig 15) With the exception of the plasma membrane found surrounding these discrete packets of saccules, no significant contamination by other sperm components was found

Immunocytochemistry using polyclonal antibodies raised against fraction 3 (anti-SE) (see section VII) was found to label the droplets of spermatozoa in the lumen of the epididymis using light microscopy, over the saccular elements of cytoplasmic droplets in situ by electron microscopy and over isolated saccular elements using electron microscopy of ultra-thin frozen sections

Fraction 4 of the gradient (0.6M/0.8 M sucrose buffer, ρ gravity 1.080/1.105) was also characterized by saccular membranous elements (Fig 16) similar in size and shape to those described for fraction 3 above However, in contrast to fraction 3 (Fig 15), the saccular elements in fraction 4 (Fig 16) were not found in discrete packets enclosed by a plasma membrane but rather were loosely arranged throughout the fraction

Although some saccular elements were found in the other three interfaces of the discontinuous sucrose gradient (i.e., fraction 1 or the pellet fraction, fraction 2 between 1.2 M and 1.0 M sucrose, and fraction 5 between 0.6 M and 0.25 M sucrose), these elements did not predominate as in fractions 3 and 4, but rather were intermixed with various other sperm fragments and components

IV POLYPEPTIDE COMPOSITION OF THE SACCULAR ELEMENTS

Polypeptide compositions of various isolated subcellular fractions from spermatozoa from initial segment and caput epididymidis were analyzed on 8 to 18% gradient polyacrylamide gels (Fig 17) Fractions S (lane S on Fig 17), which represents a crude extract of cytoplasmic droplets, contains numerous polypeptide bands at the range of 15 to 100 kD Major peptide bands were observed at 21, 41, 44.5, 68, 75 and 80 kD Subfractionation of fraction S yielded fractions 1 to R (lane 1 to R on Fig 17) In fraction 3 (Fig 17 lane 3), where saccular elements of cytoplasmic droplets were observed by electron microscopy, seven major peptide

bands of molecular masses 33, 36.3, 44.5, 68, 75, 97 and 110 kD were identified. Six of these seven polypeptides (except the 68 kD one) were more prominent than in the original crude extract (i.e. fraction S), representing an enrichment of these polypeptides. Note also that the distribution of polypeptides in fractions 3 and 4 was similar.

The transferred polypeptide bands of isolated saccular elements from fraction 3 on Western blot (Fig. 18 lane H) were comparable to those found on polyacrylamide gels (Fig. 17 lane 3). Polypeptide composition of fraction 3 isolated from caput epididymal spermatozoa (Fig. 18 lane H) was distinct from that isolated from cauda epididymal spermatozoa (Fig. 18 lane T). On immunoblots, antisera raised against isolated saccular elements from spermatozoa of the entire epididymis (anti-SE) bound most strongly to polypeptides of caput saccular elements of molecular masses 17, 36 and 44.5 kD (Fig. 18 lane H3). On the contrary, immunoblots of cauda saccular elements (Fig. 18 lane T3) showed prominent immunoreaction with anti-SE between 30 to 70 kD.

V. SUBCELLULAR DISTRIBUTION OF GOLGI APPARATUS AND LYSOSOMAL ENZYME ACTIVITIES IN EPIDIDYMAL SPERMATZOA

The activities for the enzymes galactosyltransferase (GT), sialyltransferase (ST) and acid phosphatase (AP) were first determined for the initial pellet (P) and supernatant (S) fractions of spermatozoa obtained from the initial segment and caput epididymidis (Table 1). The relative specific activities (RSA) of GT and ST were found to be more than 2-fold higher in the supernatant than in the pellet ($p < 0.005$). In the case of AP, all of the enzyme activity was localized in the supernatant ($p < 0.001$).

The supernatant fraction, enriched in cytoplasmic droplets obtained from the initial segment and caput epididymal spermatozoa, was subsequently subfractionated into 5 discrete fractions (1 to 5) by isopycnic centrifugation on a discontinuous sucrose gradient (see isolation procedure above). The enzyme activities of the corresponding subcellular fractions (1 to 5) and of the residual (R) fraction (composed of the contents of the leftover sucrose gradient) were presented, according to de Duve et al. (1975), in Fig. 19. The highest GT and ST specific activity was found in the

fraction 3 ($p < 0.005$) with the fraction 4 ($p < 0.005$) a close second. The highest fold enrichment (4-6 fold) of the specific activities of these two Golgi marker enzymes coincided with the predominance of saccular membranous elements found in fractions 3 and 4. Acid phosphatase, a lysosomal marker enzyme, was also found to be enriched in the fractions 3 ($p < 0.01$) and 4 ($p < 0.01$). However, the fold enrichment (maximum of 2-fold) of its specific activity was considerably less than that for the two Golgi marker enzymes.

The exact percent recovery of the protein content and enzyme activities for the various fractions relative to the whole supernatant fraction is presented in Table 2. The absolute values for the specific activities of GT, ST and AP in fraction 3 were determined as being 3.542 ± 0.47 nmol/min/mg protein, 1.36 ± 0.15 nmol/min/mg and 396 ± 73.6 nmol Pi/min/mg, respectively. The magnitude of the Golgi marker enzyme activities were comparable to those found in isolated rat liver Golgi apparatus (galactosyltransferase Hino et al., 1978b, Bretz et al., 1980, Bergeron et al., 1982, sialyltransferase Bretz et al., 1980).

The effect of trypsinization on galactosyltransferase activity is shown in figure 21. Under Triton X-100 free incubation conditions, the enzyme activity decreased initially to around 60% of the total activity at low trypsin concentration. The percentage of enzyme activity did not decrease any further despite the increase of trypsin concentration by another ten-fold to 100 ug/ml. On the other hand, in the presence of Triton X-100, galactosyltransferase activity decreased sharply to 17% of the total enzyme activity at a minimal concentration of trypsin. A further increase in trypsin concentration caused galactosyltransferase activity to decrease to below 5% of total enzyme activity.

Corresponding fractions, as those obtained above for caput spermatozoa, were also obtained for cauda spermatozoa and assessed for GT, ST and AP enzyme activity. Surprisingly, no significant enrichment of the Golgi marker enzyme activities was found in any of the fractions (Table 3 & Fig 20). In fact, the absolute specific activities of the two Golgi marker enzymes in the saccular element fractions 3 and 4 of the cauda epididymal spermatozoa were found to be more than 10 times lower

than that of the corresponding fractions in caput epididymal spermatozoa ($p < 0.001$). Such a drastic decrease in Golgi marker enzyme activity was not observed for acid phosphatase activity. Rather, a comparable activity profile was obtained between the corresponding caput and cauda fractions.

VI IMMUNOCYTOCHEMICAL DETECTION OF GOLGI APPARATUS MARKERS IN THE EPIDIDYMIS

1) LIGHT MICROSCOPY

Survey sections through the initial segment of the epididymis that were probed with the trans Golgi network protein (anti-TGN-38) antisera revealed an intense immunoperoxidase reaction product over the supranuclear region of the epithelial principal cells lining the tubules (Fig 22). Immunoreaction was also observed as discrete dense dots in the tubular lumen. At higher magnification, the reaction in the principal cells was discernible as a dense anastomotic network localized in the supranuclear region (Fig 23). This immunoreactive area of the cell corresponds to the region of the Golgi apparatus. No reaction was evident over the remainder of the cytoplasm or nucleus of the cell. In the tubular lumen at higher magnification, the immunoreaction was localized over discrete bodies, located along the sperm tail, which correspond to the cytoplasmic droplets (Fig 23). No staining was detectable over other regions of the tail or head of spermatozoa. Similar reactions with antisera to TGN-38, as demonstrated here for the initial segment, were found throughout the remainder of the epididymis (results not shown).

Immunoreaction with anti- β 1,4 galactosyltransferase (anti-GT) immune serum revealed an intense reaction, over the supranuclear region of the epithelial principal cells of the initial segment of the epididymis (Fig 24), similar to that shown for TGN-38 antisera. Likewise an intense reaction was observed in the sperm tail over discrete bodies which corresponded to the cytoplasmic droplets. Upon closer examination of the immunostaining reaction in the cytoplasmic droplet, it was found that the reaction was predominantly localized to one pole, coinciding with the location of the aggregated saccular elements (Fig 25). Again, no immunostaining was

detectable over other regions of the tail or head of spermatozoa. In contrast to the strong reaction observed over the cytoplasmic droplet with the IGN-38 antisera throughout the epididymis, the anti-GT reaction was undetectable in the cytoplasmic droplets of the corpus and cauda epididymidis. However, the reactivity remained over the supranuclear region of the principal cells (Fig. 27).

Anti- α 2,6 sialyltransferase (anti-ST) antibody also exclusively reacted with the supranuclear region of the principal cell and the cytoplasmic droplets within the initial segment and caput epididymidis (Fig. 26). However, as in this case with anti-GT antibody, no reaction in the above locations were observed in the remainder of the epididymis.

In summary then, regardless of the varying regional immunostaining distributions seen in the epididymis, the three antibodies against Golgi markers were found only to react with the Golgi apparatus of the principal cells and the cytoplasmic droplets of the spermatozoa.

Controls using normal rabbit sera, preimmune sera, anti-EGF and anti-insulin antibodies did not show any reaction over the epithelium or sperm in the epididymis.

2) ELECTRON MICROSCOPY

Immunocytochemistry performed at the ultrastructural level on Lowicryl embedded tissue with the three antibodies against Golgi markers (i.e., anti-Ign, anti-GT, anti-ST) confirmed the specific cellular sites and their localizations at the light microscope level. Despite the variations in immuno-gold labeling intensity observed with these four antibodies, the gold particles were present exclusively over areas containing the saccular elements of the cytoplasmic droplets of spermatozoa (Figs. 28-30). No other regions or organelles, including the plasma membrane, were labeled. As in the case of LM immunocytochemistry, the anti-GT and anti-ST antibodies were found to label more strongly the saccular elements in spermatozoa of the initial segment and caput epididymidis, while in corpus and cauda epididymal spermatozoa, significantly weaker immunoreactions were observed with these two antibodies. On the other hand, correlating with our results on LM

immunocytochemistry, anti-TCN-38 antibodies were found to label the saccular elements of spermatozoa from all regions of the epididymis

Frozen sections of isolated saccular elements (fraction 3) obtained from the initial segment and caput spermatozoa were labeled intensely with both anti-ST antibody (Fig 31) and with anti-GT antisera (Fig 32) Corresponding to our results in LM immunocytochemistry, isolated saccular elements obtained from cauda spermatozoa were only weakly labeled with these two antibodies (Fig 33)

VII IMMUNOCYTOCHEMISTRY STUDY OF THE ANTI-SE ANTIBODIES RAISED AGAINST ISOLATED SACCULAR ELEMENTS

Antibodies raised against isolated saccular elements (anti- SE) were tested on immunoblots of polypeptides of saccular elements from caput and cauda epididymidis (see section IV) To further demonstrate their specificity, immunocytochemistry on tissue sections were performed Using light microscopy, anti-SE was found to label intensely over discrete bodies of the sperm tail corresponding to the cytoplasmic droplet (Fig 34) No immunostaining was detectable over other regions of the tail or head of spermatozoa In addition, the anti-SE antibodies also label the supranuclear region of the epithelial principal cells At higher magnification, the immunostaining was confirmed to be on the cytoplasmic droplet and Golgi apparatus of the principal cells (Fig 35) Such staining pattern was consistent throughout the entire epididymis

At the electron microscope level, anti-SE labeled intensely the saccular elements of the cytoplasmic droplets in situ (Fig 36) No other regions or organelles, including the plasma membrane, were labeled Ultra-thin frozen sections of isolated saccular elements were intensely labeled with anti-SE antibodies (Fig 37) The intensity of labeling did not differ significantly between isolated fractions from caput or cauda epididymal spermatozoa

VIII LECTIN BINDING ANALYSIS OF EPIDIDYMAL SPERMATOZOA

Lowicryl embedded tissue sections at the electron microscopic level, gold-conjugated Ricinus Communis Agglutinin I (RCAI) and Helix Pomatia lectin (HPL) bound to the saccular elements of the cytoplasmic droplet as well as the adjacent plasma membrane of initial segment and caput epididymal spermatozoa (Figs 38, 39) In the presence of corresponding competitive sugars D- galactose and N-acetyl-D-galactosamine, specific lectin-gold labeling was not observed

IX ENDOGENOUS GLYCOSYLATION ASSAYS

In order to assess further the functional significance of the cytoplasmic droplet, in vitro glycosylation of endogenous polypeptides by the isolated saccular element fractions was performed as described previously for rat liver Golgi fractions (Bergeron et al, 1982, 1985) Incubation of the intact fraction 3 with UDP-[³H] galactose or CMP-[³H] sialic acid in the absence of detergent and/or any membrane perturbants, revealed that several polypeptide bands on the isolated saccular element fraction were radioactively labeled by [³H]-galactose and [³H]- sialic acid (Fig 40 lane 2 & 3) A large number of polypeptides of molecular masses between 29 and 280 kD were acceptors for glycosylation with UDP-[³H] galactose Polypeptides of molecular masses 14.4, 20.4, 34.5 and 62 kD served as major acceptors for CMP-[³H] sialic acid When comparing the two glycosylation profiles on SDS-PAGE to the Coomassie blue stained profiles, it was evident that the sialolabeled glycoprotein did not correspond to the major polypeptide bands of the fractions which were observed at 12, 44.5, 68, and 110 kD (Fig 40 lane 1)

DISCUSSION

DISCUSSION

The Golgi apparatus is ubiquitous to all eukaryotic cells and is usually defined on morphological grounds as an intracellular component of flattened membranous cisternae organized as a saccular stack with associated vesicles. The organelle has been demonstrated to be a site of regulation of protein secretion with modification reactions such as terminal glycosylation, sulphation and phosphorylation occurring to secretory and membrane proteins in transit and sorting of membrane and content proteins occurring at the exit or trans-pole of the organelle (see Pavelka, 1987 for a review). In this study we provide morphological, biochemical and immunocytochemical evidence, both in vitro and in vivo, that during spermiogenesis the Golgi elements are subsequently segregated, from other spermatid organelles, into the cytoplasmic droplet of spermatids at spermiation. These elements are retained within the cytoplasmic droplet during its distal migration along the tail of spermatozoa in the caput epididymidis and its consequent detachment from the tail beginning in the corpus and ending in the cauda epididymidis.

I ULTRASTRUCTURAL ANALYSIS OF LATE SPERMATID IN THE TESTIS

In an attempt to observe the formation of the cytoplasmic droplet, the cytoplasm of late spermatids in rats was examined morphologically by electron microscopy. It has been known for a long time that the Golgi apparatus, while morphologically identifiable in spermatids at early steps of spermiogenesis, was not found in mature spermatozoa (reviewed by Clermont et al., 1993). It appears, in our study, that the saccules of the Golgi apparatus begin to segregate from one another at step 16 and 17 of spermiogenesis. This segregation occurs concurrently with the appearance in the cytoplasm, of the loosely scattered saccular elements. Morphologically the individual saccular elements resemble the saccules of the Golgi stacks observed at earlier steps of spermiogenesis. At step 19 of spermiogenesis, the saccular elements congregate and are packaged into the droplet, while the remaining organelles (such as mitochondria, ribosomes, remnants of the endoplasmic reticulum and lipid bodies), along with the bulk of cytoplasm, are phagocytosed by the Sertoli cells to form the

residual bodies. This sequence of observations led us to investigate the hypothesis that the saccular elements of the cytoplasmic droplet are Golgi derived (Fig. 41)

II. ISOLATION OF SACCULAR ELEMENTS FROM CYTOPLASMIC DROPLETS

Using a 4-step discontinuous sucrose gradient, we developed a simple and reproducible method for the isolation of the saccular elements of the cytoplasmic droplets. Although there have been several studies reporting the isolation and biochemical characterization of the cytoplasmic droplet of spermatozoa in various mammalian species (Dott and Dingle, 1968, Garbers et al., 1970, Moniem and Glover, 1972, Roberts et al., 1976, Kaplan et al., 1984, De Vries and Colenbrander, 1990), this study is the first to isolate and biochemically and immunocytochemically characterize the saccular elements in the cytoplasmic droplets.

An essential feature of our isolation protocol is the use of a multi-step gradient which not only allows us to study the various cellular/subcellular components of the crude cytoplasmic droplet fraction, but also provides a less traumatic environment than protocols used by other investigators (Dott and Dingle, 1968, Harrison and White, 1969, Garbers et al., 1970, Roberts et al., 1976). The gradation of buoyant density avoids abrupt changes in the centrifugal forces and thus reduces the shearing forces experienced by particles as they sediment. Furthermore, as sedimentation occurs on interfaces of buffer, harsh pelleting of subcellular fractions against the tube wall is avoided, thus allowing better preservation of the morphology and integrity of the subcellular structures for further analysis.

The buoyant density of the sucrose interfaces where the saccular elements were isolated were 1.1302/1.1054 (fraction 3) and 1.1054/1.0797 (fraction 4). Interestingly, these values correspond to that of the heavy and light Golgi subfractions, respectively, from rat hepatocytes (Bergeron et al., 1982), supporting our hypothesis that the saccular elements of cytoplasmic droplets are Golgi elements.

The purity of the isolated fractions of saccular elements was examined by electron microscopy and showed that contamination by other visible cellular structures was minimal. Furthermore, polyclonal antibodies raised against the isolated fraction were found to specifically immunolabel the saccular element in situ, the absence of any cross-labeling on other cellular structures confirms the purity of our saccular element preparation.

III. DISTRIBUTION OF GOLGI APPARATUS MARKER ENZYME ACTIVITIES

Using radioactive glycosylation assays, significant fold-enrichment of galactosyltransferase and sialyltransferase, two well-known marker enzymes for the Golgi apparatus (Berger and Hesford, 1985, Roth and Berger, 1982, Roth et al., 1985, Strous et al., 1983), were identified in the isolated fractions of saccular elements from the initial segment and caput epididymal spermatozoa. The specific activities of galactosyltransferase and sialyltransferase, measured by exogenous glycosylation, have been reported in various studies using different incubation media and conditions. In general, for galactosyltransferase the reported specific activity in rat liver Golgi ranges from 3.5 to 13 nmol/mg protein/min (Hino et al., 1978a, Bretz et al., 1980, Bergeron et al., 1982), and for sialyltransferase is 8 to 14 nmol/mg/min (Bretz et al., 1980). The galactosyltransferase and sialyltransferase activities of isolated saccular element fractions were 2.1 and 1.1 nmol/mg protein/min, respectively. Although the absolute specific enzyme activities of the two Golgi marker enzymes were lower, the order of magnitudes of the values agree closely to those reported in the literature for rat liver Golgi apparatus. These data suggest that the saccular elements share in common with the Golgi apparatus the localization of GT and ST and support our hypothesis that the saccular elements are Golgi elements or Golgi derived.

Galactosyltransferase on the saccular element fraction is resistant to trypsinization in the absence of the detergent Triton X-100. The initial drop in enzyme activity at low trypsin concentration is likely due to trypsin inactivation of free galactosyltransferase present in a soluble form in the subfraction. Thereafter, despite

the increase in trypsin levels, the enzyme activity does not decrease any further. A similar kinetic pattern of galactosyltransferase activity at various trypsin concentrations is also observed in the rat liver Golgi apparatus (Fleischer, 1981). It has been proposed that such a resistance to trypsinization is related to the topologic, or spatial, arrangement of the enzyme in the Golgi saccule (Fleischer, 1981, Strous et al., 1983). According to this model, the membrane-bound galactosyltransferase is situated in the interior of the Golgi saccule, while trypsin in the medium is on the exterior side of the saccule and therefore will not be able to tamper with the enzyme. In the presence of Triton X-100, on the other hand, the integrity of the Golgi saccule membrane is disrupted and the enzyme is thus exposed for trypsin degradation. The fact that galactosyltransferase in both the droplet saccular elements and in Golgi saccules show similar kinetics in trypsinization suggests that the spatial arrangement of the enzyme relative to the membranous milieu of the two organelles are similar.

IV IMMUNOCYTOCHEMISTRY ON LIGHT AND ELECTRON MICROSCOPY

The presence of galactosyltransferase and sialyltransferase on isolated saccular elements of cytoplasmic droplets was implied by the high fold-enrichment of enzyme activities in our biochemical assays. Such findings led us to employ immunocytochemistry at the light and electron microscope levels to morphologically localize the enzymes to the saccular elements. Although the antiserum against galactosyltransferase we used has been reported to be human specific, it also contains antibodies to sugar residues (Child et al., 1986, Watzele et al., 1991). For this reason, we used, in addition, antibodies to protein affinity-purified anti- α 2,6 sialyltransferase and antisera to TGN 38 for our study. Positive immune reactions were demonstrated with all these antibodies on the saccules of the droplet, the Golgi region of epithelial principal epididymal cells and the isolated saccular elements, providing further evidence that saccular elements from cytoplasmic droplets share similarities with the Golgi apparatus.

The plasma membrane of the head and tail of spermatozoa was not reactive to any of the antibodies. The absence of immunolabeling with galactosyltransferase antibodies on spermatozoa plasma membrane is of particular interest. Previous investigations have reported the presence of sperm-surface galactosyltransferase activity in the head or at least the acrosome of mouse spermatozoa (Shur and Roth, 1975, Durr et al., 1977, Shur and Hall, 1982a & 1982b, Lopez et al., 1985, Scully et al., 1987, Lopez and Shur, 1987, McLaughlin and Shur, 1987, Scully et al., 1987, Scully and Shur, 1988, Shur and Neely, 1988, Benau and Storey, 1988, Fayrer-Hosken et al., 1991, Miller et al., 1992). Considerable evidence has been presented that surface galactosyltransferase in the mouse spermatozoon mediates sperm-egg recognition (Shur and Hall, 1982a & b, Lopez et al., 1985, Scully et al., 1987, Hathaway and Shur, 1988, Shur and Neely, 1988). Our results do not confirm the presence of galactosyltransferase on sperm plasma membrane. However, whether such a discrepancy can be explained by the low specificity/affinity of the antibodies we used to the particular sub-type of galactosyltransferase, if it exists, on sperm plasma membrane remains to be answered.

Our negative result concurs with the findings of Tulsiani et al. (1990) who also found no galactosyltransferase activity in plasma membrane preparations of human epididymal sperm. It is of interest that in one segment of their investigation dealing with the obtention of contaminant-free human spermatozoa from a discontinuous percoll gradient, the authors reported that nearly all of the measurable galactosyltransferase activity was found at the top of the gradient consisting of seminal plasma, unidentified vesicles and other vesicles such as the cytoplasmic droplets. This latter observation, based on the results of our study, suggests that the source of galactosyltransferase in their light fraction could have been emanating from the saccular elements of the cytoplasmic droplet. We further suggest the possibility that in the initial investigation reporting the presence of galactosyl- and sialyltransferase in the mouse sperm plasmalemma (Durr et al., 1977, Shur and Bennett, 1979), the washed sperm fractions that were found enriched in the two glycosylation enzymes could have been contaminated with enzymes originating from

the saccular elements of the cytoplasmic droplet, this hypothesis would require experimental proof

V. ENDOGENOUS GLYCOSYLATION BY SACCULAR ELEMENTS

We have demonstrated the presence of galactosyltransferase and sialyltransferase on the saccular elements by immunocytochemistry. Radiobiochemical assays showed that those two enzymes were active *in vitro*. The functional significance of the saccular elements was further evaluated by two approaches in the present study. In the first, endogenous glycosylation was attempted with both UDP-[³H] galactose and CMP-[³H] sialic acid as glycosyl donors for galactosyltransferase and sialyltransferase, respectively. Incorporation of nucleotide sugars by both galactosyltransferase and sialyltransferase were observed in endogenous polypeptides. The incubations were performed in the absence of cytosol or any membrane perturbants (detergent and, for CMP-[³H] sialic acid, high concentration of Mn²⁺) (Bergeron et al., 1982). These findings thus suggest that, besides the relevant glycosyltransferase and endogenous polypeptide acceptors, the nucleotide sugar transporters were functionally active in cytoplasmic droplet saccular elements.

The second method of assessing the ability of glycosylation *in situ* of the saccular elements was by lectin staining assays. Both RCA I and HPL staining revealed high concentrations of the cognate sugar ligands (galactose and N-acetyl galactosamine, respectively) over saccular elements of the cytoplasmic droplet. A striking observation is that the associated plasma membrane was also reactive to lectin binding. Complementing these results was our earlier observations revealing spot associations of saccule membranes with each other as well as with the adjacent plasma membranes. Such membrane associations were also identified previously by Friend and Heuser (1981) as fusion intermediates and may signify a site of exchange. These results provide circumstantial evidence which is consistent with transport of glycoconjugates from the saccular elements to the adjacent plasma membrane. However, definitive proof for such a unique transport system will ultimately depend upon the ability to follow the newly derived glyco-conjugates from the saccular

elements to the associated plasma membrane of epididymal spermatozoa in situ

VI SIGNIFICANCE OF THE MATURATION-DEPENDENT DIMINUTION OF GLYCOSYLTRANSFERASES ACTIVITIES IN SACULAR ELEMENTS OF CYTOPLASMIC DROPLETS

During epididymal transit, the cytoplasmic droplets are displaced distally along the mid-piece of the sperm tails and eventually begin to detach from the sperm tails in the cauda epididymidis. In this study we processed the sacular elements from droplets of caput (including the initial segment, proximal and distal caput) and cauda (including proximal and distal cauda) epididymal spermatozoa separately. Using electron microscopy, the morphological appearance of the sacular elements in the two different regions of the epididymis were similar, whether they were observed in situ or in isolated subcellular fractions. On comparison of their polypeptide compositions on SDS-PAGE, however, subtle dissimilarities were observed. Such an observation was further illustrated by the differences in labeling patterns on immunoblots with anti-SE anti-sera. These findings may imply that the sacular elements are undergoing modifications in their peptide composition during spermatozoa epididymal transit. Indeed, as we found on biochemical and immunocytochemical analyses, sacular elements from caput and cauda epididymal spermatozoa do have significance differences.

First, contrary to sacular elements from caput epididymal spermatozoa, those isolated from cauda epididymal spermatozoa show a much lower fold-enrichment of galactosyltransferase and sialyltransferase. In fact, the absolute specific activities of the two enzymes in the sacular elements of cauda epididymal cytoplasmic droplets are more than ten times lower than that of the corresponding fractions from caput spermatozoa. Consistent with such findings were our observations that the immunolabeling with anti-GT antibodies on sacular elements from cytoplasmic droplets of cauda epididymal spermatozoa was also significantly lower than on those of caput epididymal spermatozoa. Our findings support the hypothesis that the contents of cytoplasmic droplet sacular elements undergo some modification during spermatozoa epididymal transit. Moreover, such modifications appear to be selective.

As the levels of galactosyltransferase and sialyltransferase decrease, levels of TGN-38, as analyzed by immunolabeling, do not differ significantly in the sacular elements from caput to cauda epididymidis. The mechanism and significance of such modifications remain to be elucidated.

The presence of glycosyltransferases in epididymal fluid (Durr et al, 1977, Shur and Bennett, 1979, Hamilton, 1980, Lopez et al, 1985, Tulsiani, 1993) and their differences in fold enrichment in various regions (Bernal et al, 1980, Hamilton, 1980, Tulsiani et al, 1993) have been well documented. Tulsiani et al (1993) recently reported that although the majority of glycosyltransferases activities were present in soluble form in the epididymal fluid, 10-20% of the total activities are associated with epididymal spermatozoa. They further demonstrated that sialyltransferase activity associated with spermatozoa gradually decrease as spermatozoa moved from the rete testis to the distal cauda epididymidis. This drop in enzyme activity complements the findings of Bernal et al (1980), who demonstrated that caput, but not cauda, spermatozoa can replace disialo-fetuin as an acceptor of sialic acid in sialyltransferase assays. It also complements the data of other investigators who found that the amount of sialic acid bound to the sperm membrane increases rapidly to reach a maximum in the corpus epididymidis (Prasad et al, 1973, Laporte et al, 1975).

The consistency of the diminution of glycosyltransferases activities associated with whole spermatozoa and sacular elements of cytoplasmic droplets supports the view that perhaps the sacular elements represents the main source of glycosyltransferases in epididymal spermatozoa. Considering further the fact that the diminution of glycosyltransferase activities in cytoplasmic droplets coincides with the maturation of spermatozoa during their epididymal transit logically leads one to speculate a possible biological role for the cytoplasmic droplet in epididymal spermatozoa maturation.

VII THE IDENTITY OF THE SACCULAR ELEMENTS IN CYTOPLASMIC DROPLETS

Early work by Dott and Dingle (1968) compared the activity of acid phosphatase in isolated cytoplasmic droplets and spermatozoa of the bull and ram and found that the enzyme activities in spermatozoa with cytoplasmic droplets were significantly higher than that in spermatozoa devoid of droplets. On this basis, they suggested that the cytoplasmic droplets were modified lysosomes. In our study, we evaluated the specific activity of acid phosphatase on the isolated saccular elements. Although localized in the saccular element fraction, the fold enrichment of acid phosphatase specific activity was low and was dispersed throughout the fractions of the entire discontinuous gradient. Our results thus dispute the idea that the cytoplasmic droplet is a modified lysosome. Previous investigation supporting our claim includes in situ EM cytochemistry for acid phosphatase using a number of substrates (Hermo et al., 1988) which failed to show any reactivity in the cytoplasmic droplet.

Other possibilities of the origin of the saccular elements of the cytoplasmic droplet have been considered. In a recent work by our group (Oko et al., 1993), antisera specifically against the endoplasmic reticulum (Louvard et al., 1982) and an endoplasmic reticular membrane protein calnexin (Wada et al., 1991) failed to react with the cytoplasmic droplets of epididymal spermatozoa or late (step 19) spermatids in testis. In addition, tracer studies for fluid phase or adsorptive endocytosis by injection of cationic ferritin, anionic ferritin or horse-radish peroxidase to lumen of the rete testis showed no deposition to the saccular elements of the droplets (Oko et al., 1993). These results suggest that the saccular elements are probably unrelated to the endoplasmic reticulum and, furthermore, not a component of the endocytic apparatus.

A tentative identification of the saccules of the droplet as being Golgi derived (Fig. 41) was supported by our results on the morphological, biochemical and immunocytochemical characteristics of the organelle, and on their ability to glycosylate endogenous substrates. The presence of acid phosphatase activity within the saccular elements of the droplet does not dispute a Golgi origin, as it has been

documented that the enzyme is normally present within the saccules of the Golgi apparatus (Smith and Farquhar, 1966, Bertolini and Hassan, 1967, Friend and Farquhar, 1967, Cheetham et al, 1971, Nyquist and Mollenhauer, 1973, Farquhar et al, 1974, Hino et al, 1978a & 1978b) Further investigations that could serve to support our hypothesis may include the use of the antibodies we raised against the saccular elements, with proper affinity-purification, as probes to trace if the origin of some of the polypeptide components are related to the Golgi apparatus during spermiogenesis in the testis

This study raises two major related biological issues First, the Golgi apparatus in vivo can be modified structurally into an unstacked arrangement of saccular elements which still have the capacity to endogenously glycosylate proteins Secondly, epididymal spermatozoon may have an intrinsic maturational capacity to glycosylate proteins by utilizing such Golgi-derived saccular elements These issues and related questions will be discussed in the context of what is already known about naturally occurring and experimentally induced modifications of the Golgi apparatus and about the acquisition of glycosylated proteins on the plasmalemma during epididymal maturation of the spermatozoa

VIII MODIFICATION OF THE PLASMA MEMBRANE OF SPERMATOZOA DURING EPIDIDYMAL TRANSIT AND THE POTENTIAL BIOLOGICAL ROLE OF THE SACULAR ELEMENTS

During spermatozoa epididymal transit, it is firmly established that their plasma membrane is modified by the addition or alteration of glycoproteins (Olson and Hamilton, 1978, Holt, 1980, Brown et al, 1983, Olson et al, 1987, Tulsiani et al, 1993, see also reviews by Eddy, 1988, Orgebin-Crist, 1987, Yanaginachi 1988, Robaire and Hermo, 1988) However, the outstanding question remains as to how such modifications are accomplished

Olson and Hamilton (1978) suggested three possibilities of how the plasmalemma of epididymal spermatozoa could acquire "new" glycoproteins 1) the protein could be secreted by the epididymal epithelium and subsequently bind to the sperm surface. 2) some unmasking or modification event, that is, extrinsic glycosylation, occurs to

pre-existing membrane proteins, 3) the protein is stored within spermatozoa and, at some point during maturation, a specific intrinsic triggering event causes it to be inserted in the lipid bilayer of the extracellular space

1 ACQUIREMENT OF NEW PROTEINS SECRETED BY EPIDIDYMAL EPITHELIUM

Precedents regarding the first possibility are many. For example, acidic epididymal glycoprotein (Lea et al., 1978), forward motility protein (Brandt et al., 1978, Acott and Hoskins, 1981), acrosome stabilizing factor protein (Thomas et al., 1984), His proteins (Rifkin and Olson, 1985), SGP-2 (Sylvester et al., 1984, Hermo et al., 1991b), and androgen dependent proteins (Jones et al., 1980, Brown et al., 1983, Brook et al., 1986, Brooks 1987 a,b, Moore et al., 1990) have all been shown to be secreted by various regions of the epididymal epithelium and then to become associated to the sperm surface. In fact Klinefelter and Hamilton (1985) showed by perfusion organ culture of proximal and distal rat caput epididymal tubules that at least five L-[³⁵S] methionine labeled polypeptides are synthesized and secreted by these cultured tubules and become associated with luminal sperm. However, in all the above cases the degree or mechanisms of association (e.g. receptor mediated) of these secreted proteins with the sperm membrane are unknown, it is not known whether these proteins are integral or surface components of the plasmalemma. Furthermore, these epididymal secreted proteins do not account for all of the glycoproteins implicated to be acquired by the sperm plasmalemma during epididymal transit. It is thus prudent to also evaluate the other two possibilities of how new glycoproteins are acquired, which suggest an extrinsic mechanism to glycosylate pre-existing plasmalemma proteins or to incorporate stored glycoproteins into the plasmalemma.

2 MODIFICATION OF SPERMATOZOA VIA AN EXTRINSIC MECHANISM

The second possibility of extrinsic glycosylation assumes that there would be glycosyltransferases secreted into the epididymal lumen by either the seminiferous or epididymal epithelium. In considering this hypothesis, Hamilton (1980) evaluated the

kinetics and activity of one of the glycosyltransferases, namely, N-acetylglucosamine galactosyltransferase, in fluids from rat rete testes and epididymis, and concluded that the enzyme is likely to be synthesized and secreted by the testis. Furthermore, the specific activity of this enzyme was higher in the rete testis than in the caput epididymidis. This abrupt decrease in enzymatic activity, which is coincident with the time of acquisition of fertilizing ability by spermatozoa in the rat, led Hamilton (1980) to speculate that this enzyme has a relationship to spermatozoon maturation. Such a view is shared by a number of authors who reported that sperm surface glycoconjugates are modified relatively early in epididymal transit (Durr et al., 1977, Orgebin-Crist, 1987, Hammerstedt and Parks, 1987, Eddy, 1988, Yanagimachi, 1983).

A recent study by Tulsiani et al. (1993) supported the hypothesis that extrinsic glycosylation contributes to the appearance of new glycoproteins on plasma membrane of epididymal spermatozoa. They provided evidence that a transient binding of sialyltransferase and fucosyltransferase to endogenous sugar acceptor molecules on the sperm surface occurs (both enzymes showed a high activity in caput spermatozoa). These enzymes subsequently release from the sperm surface in the distal part of the epididymis, after sialylation and fucosylation of the acceptor molecules. As acknowledged by these investigators, additional proof for this mechanism lies in the detection of endogenous nucleotide sugar donors. Furthermore, an immunolocalization of these glycosyltransferases onto the sperm membranes is imperative because the exact location of these enzymes cannot be precisely determined by biochemical means.

3 THE POSSIBILITY OF INTRINSIC GLYCOSYLATION OF PLASMA MEMBRANE PROTEINS BY THE SACULAR ELEMENTS OF CYTOPLASMIC DROPLET

Modification of spermatozoa plasma membrane via an intrinsic mechanism implies, to a certain extent, an autonomous characteristic of spermatozoa in maturation. Such an issue was first raised by Orgebin-Crist (1969) who pointed out that convincing evidence is still lacking as to whether the factors governing the maturation process of spermatozoa are intrinsic to the spermatozoa themselves and just require time, or whether spermatozoa must pass through most of the epididymis in order to mature.

The work by Silber et al (Silber, 1988, Silber et al, 1988, Silber, 1989a, 1989b, Silber et al, 1990, Asch and Silber, 1991) supports the notion that a functioning epididymis is not a *sine qua non* for spermatozoa maturation. Using human subjects with congenital absence of the vas deferens as a model, they showed that spermatozoa aspirated from vasa efferentia and different regions of the epididymis can result in pregnancy by in vitro fertilization in 31% of cases (Silber et al, 1990). Moreover, in human subjects with bilateral epididymal obstruction, who had undergone microsurgical anastomosis of vas deferens to epididymal tubule proximal to the site of obstruction (vasoepididymostomy) to allow spermatozoa to bypass the blockage, pregnancy by natural intercourse can be achieved in 31% to 56% of cases (Silber, 1989).

Spermatozoa released from seminiferous tubules have the majority of their cytoplasm, except for the saccular elements, segregated into the residual bodies which are phagocytosed by the Sertoli cells. This phenomenon implies a lack of the regular machinery, in particular of a functioning rough endoplasmic reticular system, required for extensive biosynthetic processes in the cells. In view of this, it is not surprising that, up to now, most investigators are in favor of the extrinsic mechanism in rendering spermatozoa maturation.

Hermo et al (1994) established four criteria to be met in order for intrinsic glycosylation of spermatozoa plasma membrane to be feasible. First, epididymal spermatozoa would have to have a compartment in which to store functionally active glycosyltransferases since new proteins are no longer synthesized at this stage of maturation. Second, the proteins or substrates (i.e. endogenous acceptors) to be glycosylated would also have to be in plentiful supply. Third, if the glycosyltransferases were to be stored in a membrane compartment, as they are in the saccules of the Golgi apparatus, a functionally active nucleotide sugar transporter system would have to be present. Finally, there would have to be a mechanism for the transport of newly glycosylated acceptors to and into the plasma membrane of epididymal sperm. The saccular element system in cytoplasmic droplets analyzed in this study appears to meet the above criteria.

The exclusive localization of glycosyltransferase in the spermatozoon, as established by biochemical analysis and confirmed by immunocytochemistry, satisfies the first criterion. Our endogenous glycosylation assays showed convincingly that labeled sugars were transferred into several endogenous proteins ranging in molecular masses from 10 to 145 kD. Moreover, as the incubations were done in the absence of detergents and membrane perturbants, nucleotide sugar transport through the saccular membrane most probably is operating. Finally, the high concentrations of the cognate sugar ligands over the saccular elements and the adjacent plasma membrane, and their associations with each other, as we observed on electron microscopy, provides circumstantial evidence on the transport of glycoconjugates from the saccular elements to the adjacent plasma membrane.

In conclusion, the saccular elements in cytoplasmic droplets of epididymal spermatozoa are likely to originate from the Golgi apparatus (Fig 41). Its ability to glycosylate endogenous proteins may represent an example of an intrinsic mechanism whereby spermatozoa acquire new glycoproteins. The contribution of such a potential way of plasma membrane modification in spermatozoa epididymal maturation demands further investigations.

SUMMARY

SUMMARY

The cytoplasmic droplet of epididymal spermatozoa is a small localized outpouching of cytoplasm of the sperm's tail, found in all mammalian species studied. Its biological significance is unknown. Electron microscopy revealed flattened membranous elements, termed saccular elements, as the near exclusive membranous component of the droplet. In this study, we analyzed ultrastructurally the cytoplasm of spermatids during spermiogenesis in an attempt to determine the origin of the saccular elements in the cytoplasmic droplets. The droplet first appeared just prior to the detachment of late step 19 spermatids from the Sertoli cell in the seminiferous tubules of the testis. The loss of Golgi saccular stacking in late spermatids (steps 17-18) was coincident with the dispersion of flattened membranous saccular elements in the cytoplasm of these cells and their segregation into the cytoplasmic droplet of step 19 spermatids. This sequence of events suggested that the saccular elements were Golgi derived. To analyze whether these saccules share biochemical properties with the Golgi apparatus, the saccular elements of the cytoplasmic droplet were isolated by subcellular fractionation and their polypeptide composition was analyzed using polyacrylamide gel electrophoresis. Immunocytochemical studies at the light and electron microscopic levels revealed the saccular elements of the cytoplasmic droplet as the exclusive site of reactivity for Golgi apparatus markers TGN 38, α 2,6 sialyl-transferase and β 1,4 galactosyltransferase. Antibodies raised to the isolated fraction immunolabeled saccules of the droplet in situ as well as Golgi apparatus in somatic cells of the epididymis and other tissues. In addition, in situ lectin binding assay on electron microscopy demonstrated the presence of D-galactose and N-acetyl galactosamine constituents in the saccules within the droplet and also in the adjacent plasma membrane. Using radioactive glycosylation assays with exogenous acceptors, the isolated saccular fraction was found to be enriched in galactosyltransferase and sialyltransferase activities. In endogenous glycosylation assays, glycosyl-modification of endogenous peptides of 62, 34.5, 20.4 and 14.4 kD by sialic acid and a large number of polypeptides of molecular masses between 29 and 280 kD by galactose was identified. In view of the close proximity of the saccular elements to the

spermatozoon plasma membrane, the selectivity of lectin binding to these sites and the evidence that these saccules are Golgi elements capable of endogenous glycosylation, this study suggests that the saccular elements of the cytoplasmic droplet have a potential role for modifying the plasma membrane of spermatozoon during transit through the epididymis

**PRINCIPAL CONTRIBUTIONS
OF
THIS WORK**

PRINCIPAL CONTRIBUTIONS OF THIS WORK

- I) First morphological analysis, at the electron microscope level, of the dissociation of the Golgi apparatus and the fate of its components in late spermatid cytoplasm during spermiogenesis
- II) Isolation and purification of the saccular elements of the cytoplasmic droplets from rat epididymal spermatozoa that can be used for biochemical and morphological analysis
- III) Demonstration of the polypeptide components of the saccular elements and their subtle differences between those from caput versus cauda epididymal spermatozoa
- IV) Production of specific antibodies against components of the saccular elements. Such antibodies may be further purified and used as probes to trace the origin of various components of the saccular elements during spermiogenesis
- V) Demonstration of the localization of Golgi markers enzymes, galactosyltransferase and sialyltransferase activities to saccular elements using biochemical assays
- VI) Providing evidence suggesting that the topology of galactosyltransferase on the saccular elements is similar to that found in rat liver Golgi apparatus
- VII) Demonstration of the ability of the saccular elements to glycosylate peptides endogenously using radioautography

- VIII) Demonstration of the presence of Golgi markers enzymes, galactosyltransferase, sialyltransferase and trans Golgi network protein (TGN-38) in the cytoplasmic droplet saccular elements using immunolabeling at light and electron microscopic level
- IX) Demonstration of the maturation-dependent modifications in activities of Golgi marker enzymes, galactosyltransferase and sialyltransferase on saccular elements of cytoplasmic droplets during spermatozoa epididymal transit
- X) Demonstration of the presence of lectin binding saccharides, D-galactose and N-acetylgalactosamine, over the saccular elements and adjacent plasma membrane, suggesting the possibility of transport of glycoconjugates from saccular elements of plasma membrane
- XI) Proposal of the hypothesis that the saccular elements of spermatozoal cytoplasmic droplets are Golgi saccules that may have a functional significance in epididymal sperm maturation

FIGURES AND TABLES

Fig. 1. Diagram illustrating the position of the cytoplasmic droplet of the spermatozoon in the testis and different regions of the excurrent duct system and its suggested ultimate fate. The bulk of the late spermatid's cytoplasm, referred to as the residual body, detaches and is phagocytosed by Sertoli cells lining the seminiferous epithelium. In the testis and ductuli efferentes, the droplet surrounds the neck region of the flagellum (arrowhead), while in the caput epididymidis, the droplet appears near the junction of the middle and principal piece of the flagellum (arrow). The droplet shows a lateral displacement in the corpus epididymidis (curved arrow), suggesting that it is being shed, while in the cauda, most spermatozoa are devoid of droplets. While intact droplets are not conspicuous, their contents are found free in the lumen as well as within the endocytic organelles of the epithelial clear cells. This suggests that once droplets are released from spermatozoa, they break up in the lumen, liberating their contents, which appear to be endocytosed selectively by clear cells, presumably to be degraded within their numerous secondary lysosomes (L). (Reproduced from Hermo et al., 1988)

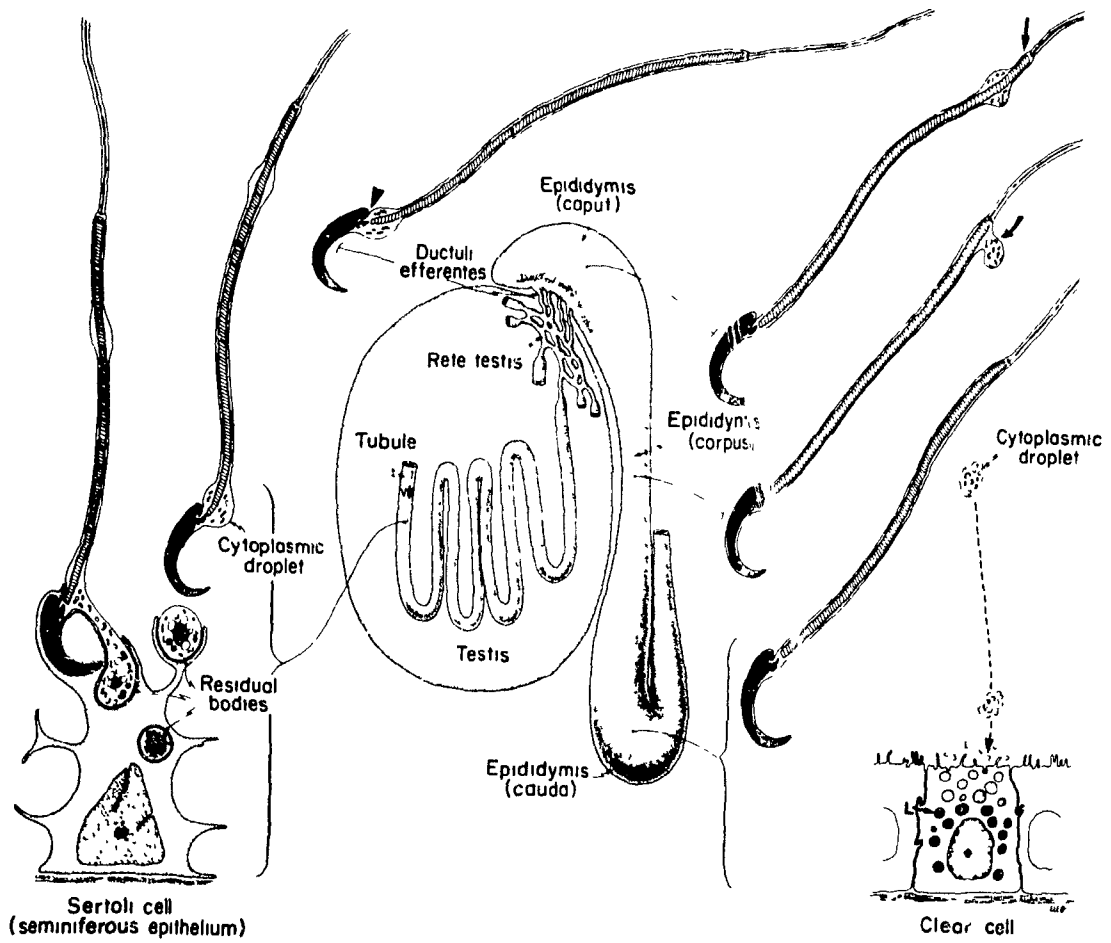


Fig. 1

Fig. 2. Electron micrograph (EM) showing a cross section through the tail of a spermatozoon at the level of the cytoplasmic droplet in the lumen of the initial segment of the epididymis. The saccular elements (S) occupy one pole of the droplet, they appear as flattened elements which may be fairly straight (short arrows) or C-shaped (curved arrows) in appearance. They may be closely stacked together (open arrow) or loosely distributed. The edge of one of these elements is occasionally closely associated with another saccular element (long arrows). There is also a close approximation of the edge of these saccular elements with the plasma membrane of the droplet (arrowheads). m mitochondrial sheath, ODF outer dense fibers, A axoneme, v vesicles $\times 45,000$

Fig. 3. High power electron micrograph of the saccular elements within the cytoplasmic droplet of a spermatozoon in the lumen of the initial segment. The flattened or C-shaped saccular elements (curved arrows) show areas of closely approximation amongst one another (short arrows) but large gaps between such elements are also evident (asterisks). The edges of the saccular elements often contact one another with some fine filamentous dense material intervening (long arrows). In addition, the saccular elements come in close proximity to the plasma membrane (arrowheads) $\times 75,000$

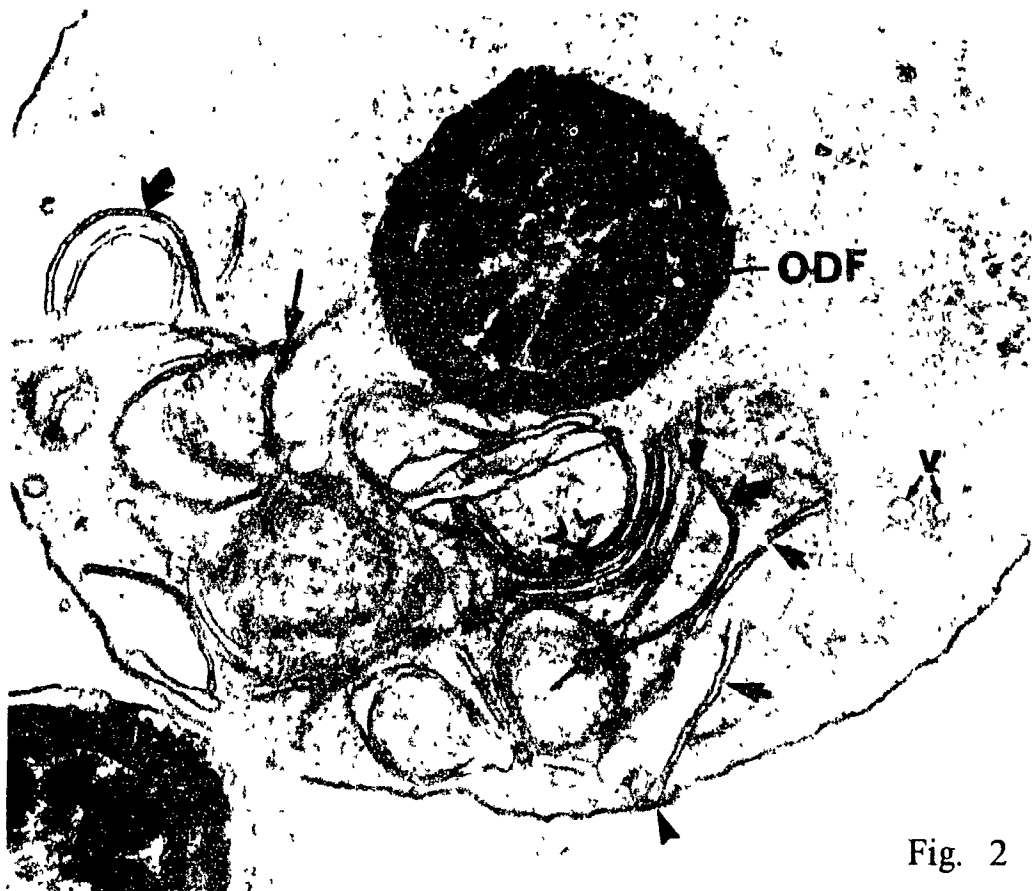


Fig. 2



Fig. 3

Fig. 4. Tangential section through the cytoplasmic droplet of a spermatozoon in the lumen of the initial segment of the epididymis. Numerous flattened saccular elements appear in the droplet with a fairly straight (short arrows) or C-shaped (long arrows) appearance, some are tightly packed together (curved arrows) while others are loosely distributed. Grazing sections through the surface of the saccular elements (asterisks) reveal a disc or plate like appearance. This confirms the saccular, as opposed to tubular, nature of the saccular elements. Some of the saccular elements present small vesicular blebs on their surface (arrowheads). Small vesicles (v) also appear in the droplet. ODF outer dense fibers, m mitochondria x 55,000

Fig. 5. Electron micrograph of a section of the cytoplasmic droplet showing numerous saccular elements. Besides adopting a C-shaped appearance (arrows), on appropriate sections, the saccular elements may appear as spherical bodies enclosed by a double membrane (curved arrows). Often a significant portion of the saccular elements is in contact with the plasma membrane (arrowheads), although fusion between the two structures are never observed x 45,000



Fig. 4

Fig. 5

Fig. 6. Electron micrograph showing the cytoplasm of an early spermatid. An intact Golgi apparatus juxtaposes to the acrosome (ACR) and the nucleus (Nu) is shown. In early spermatids the Golgi saccules are in an ordered, stacked-like appearance x 38,000

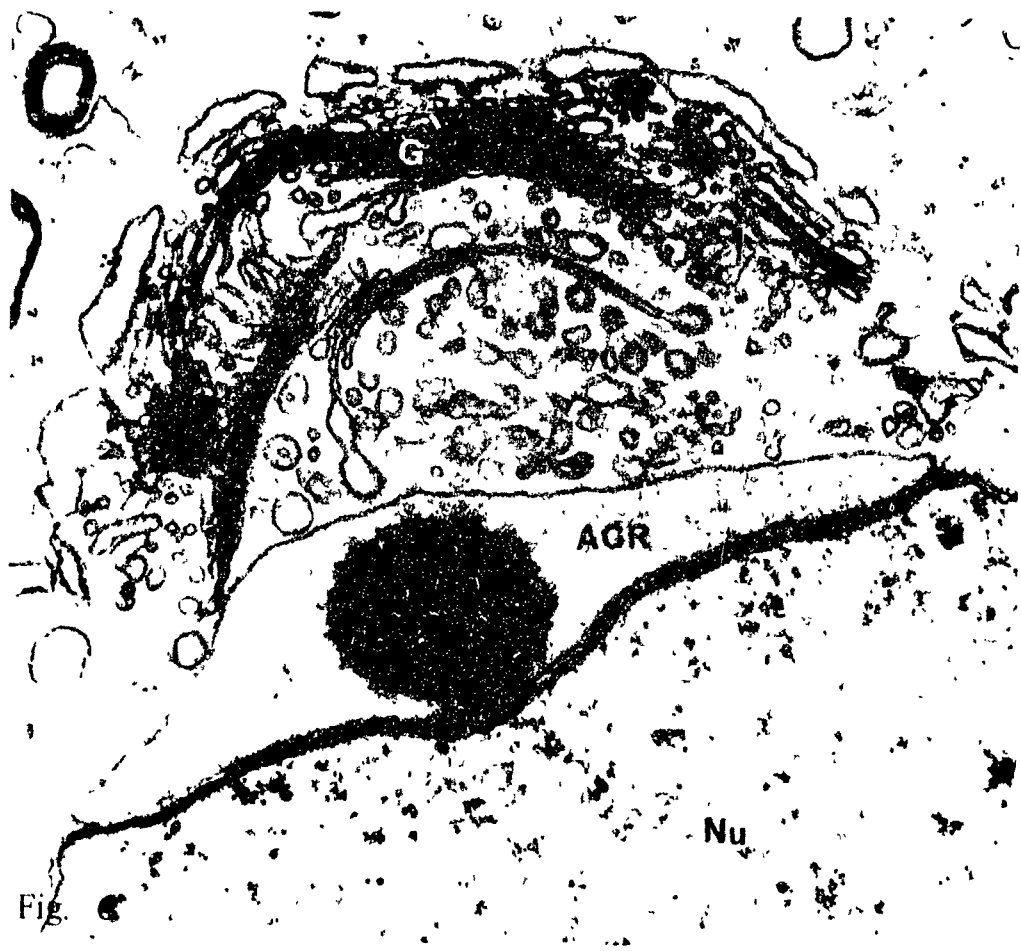


Fig. 6

Fig. 7. Electron micrograph showing the Golgi apparatus of a step 10 spermatid. At this step the Golgi is spherical and large. Several stacks of saccules (S) forming the compact zone (CZ) are present and connected to each other by tubules in the so-called intersaccular connecting non-compact zones (NCZ). On the cis face, the cis-element (C) is visible subjacent to which are many short, flattened membranous saccules of the compact zone. At the edges of the saccules, dilated tubules (I) are present serving to connect saccules of one stack with those of an adjacent stack. On the trans face, many tubulo-vesicular profiles (v) of different sizes are present including what appear to be detached saccular elements (arrows). Cisternae of endoplasmic reticulum (ER) appear as large dilated irregularly shaped elements containing a flocculent material, they appear on both the cis and trans face of the Golgi stacks as well as in the surrounding cytoplasm. $\times 35,000$

Fig. 8. Cytoplasm of a step 16 spermatid. Stacks of Golgi saccules (S) are less elaborate and conspicuous, and enveloped by small vesicles (v). It is not until this step that some flattened saccular elements (arrows) are seen, they are seen at a distance from the Golgi apparatus, which is no longer spherical. Dilated cisternae of endoplasmic reticulum (ER) are found close to the Golgi stacks as well as in the surrounding cytoplasm. An anastomotic tubular network (curved arrow) is present on the trans face of the Golgi stacks. $\times 42,000$



Fig. 7

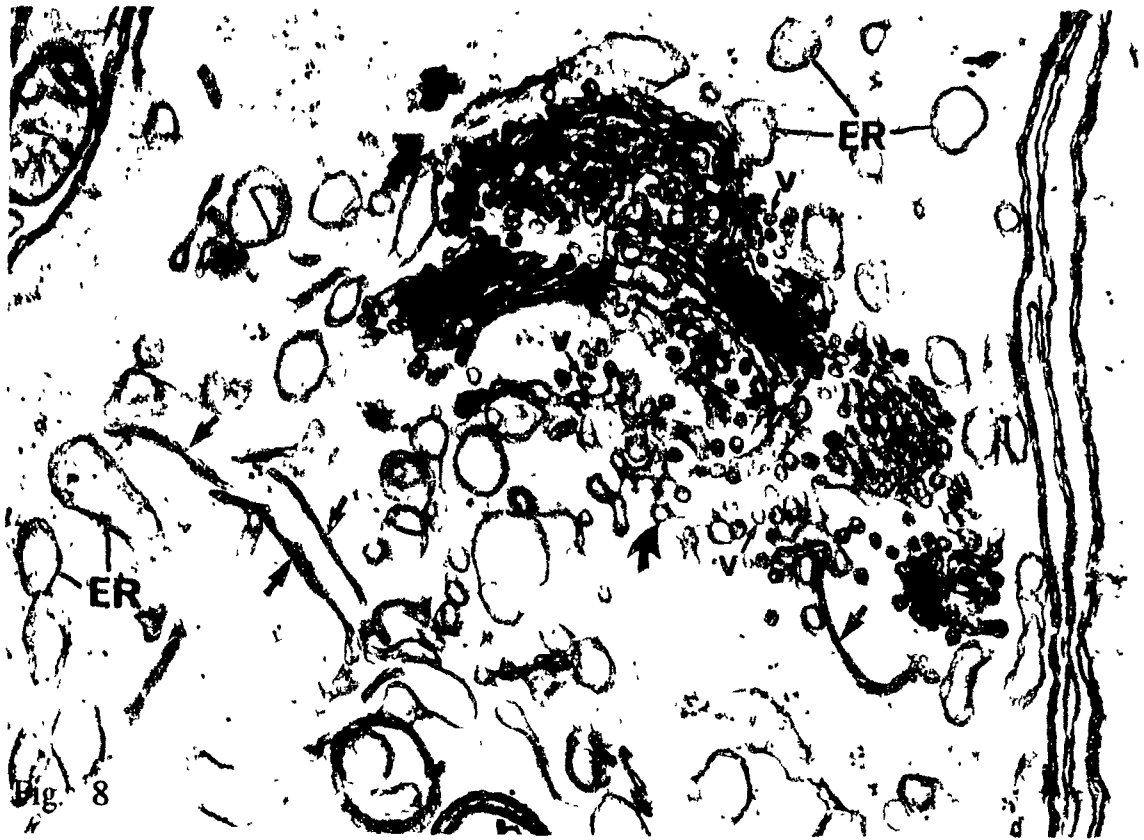


Fig. 8

Fig. 9. Cytoplasm of a step 17 spermatid. Flattened saccular elements (curved arrows) are randomly distributed in the cytoplasm. Elements of the endoplasmic reticulum (ER) are dilated, irregular in appearance and contain a flocculent material. Cross section through the tail reveals mitochondria (m) forming the mitochondrial sheath, 9 coarse outer dense fibers (ODF) and a centrally located axoneme (A). Inset: Small area of cytoplasm of a step 17 spermatid showing the remnants of the Golgi apparatus. Small vesicular profiles (v) and only a few short flattened saccular elements (curved arrows) not tightly stacked together are evident in the vicinity of cisternae of endoplasmic reticulum (ER). A few C-shaped saccular elements (arrows) are also seen in this aggregate, they appear as spherical bodies enclosed by a double membrane when cut across both arms (arrowhead) x 45,000

Fig. 10. Cytoplasm of a step 18 spermatid showing the remnants of the Golgi apparatus at higher magnification in material treated with tannic acid. Several flattened saccular elements (curved arrows) are present in an aggregate also containing dilated cisternae of endoplasmic reticulum (ER) and spherical bodies enclosed by a double membrane (arrowheads), the latter correspond to appropriate sections through saccules which are curled and show a C-shaped appearance. These structures including a few small vesicles (v) are embedded in a cytoplasmic amorphous moderately dense material (asterisks). A similar material also appears sandwiched between flattened saccular elements and ER cisternae (arrow) x 50,000

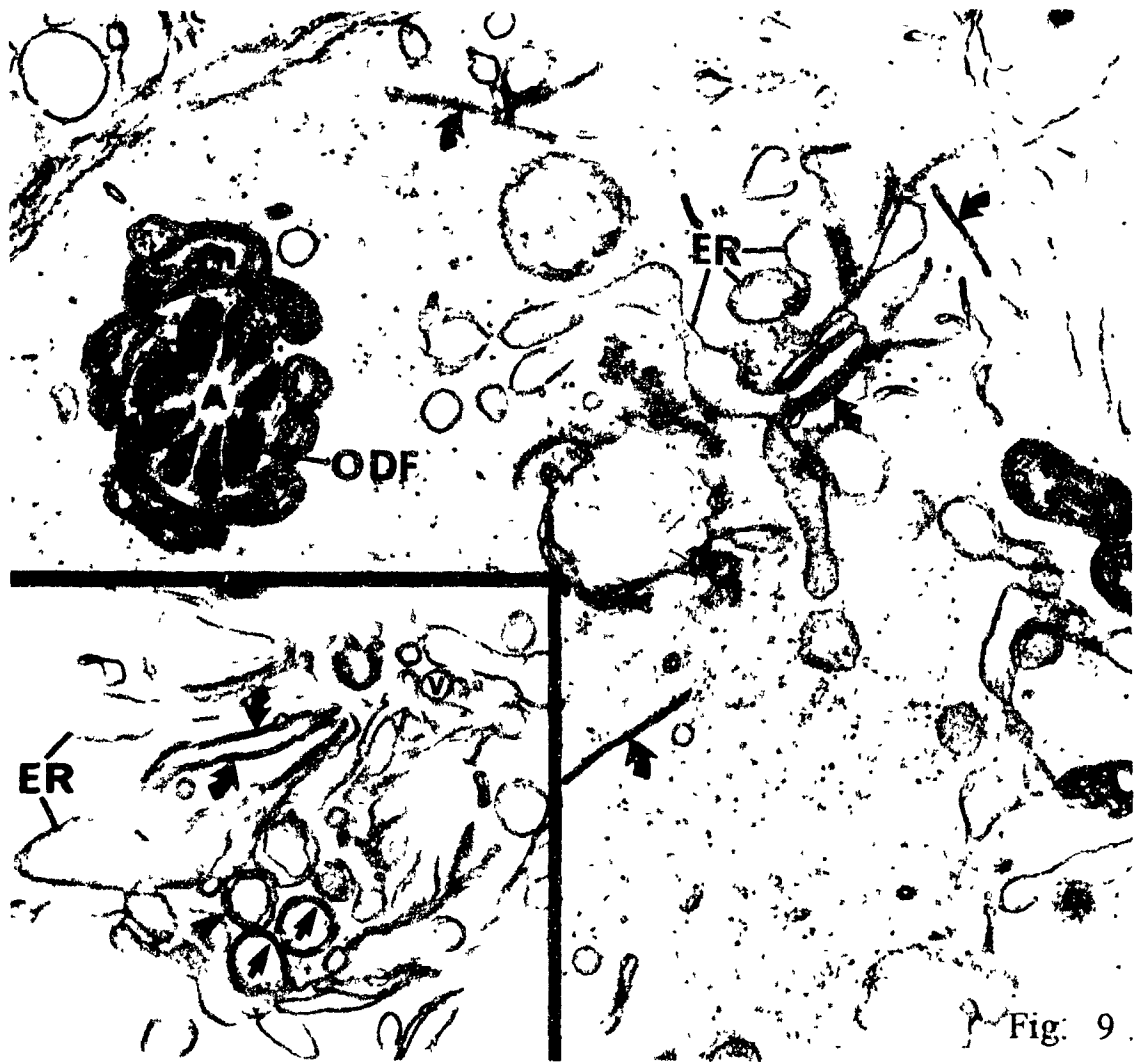


Fig. 9

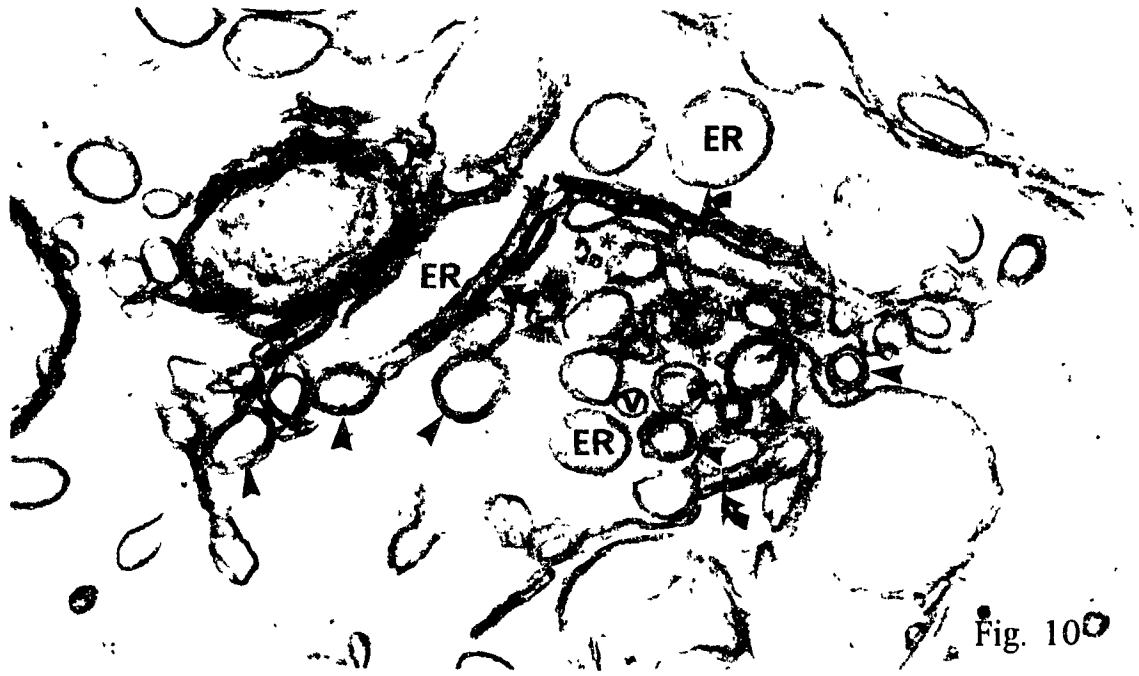


Fig. 10

Fig. 11. Cytoplasm of an early step 19 spermatid. In the upper corner a small remnant of the Golgi apparatus (arrowheads) is present containing saccular elements (arrows) and small vesicles. Randomly distributed throughout the cytoplasm are many short flattened saccular elements (arrows), some of which also appear at a close distance to the Golgi remnant. Cisternae of the endoplasmic reticulum (ER) are plentiful and appear as dilated elements connected by short bridges. x 35,000

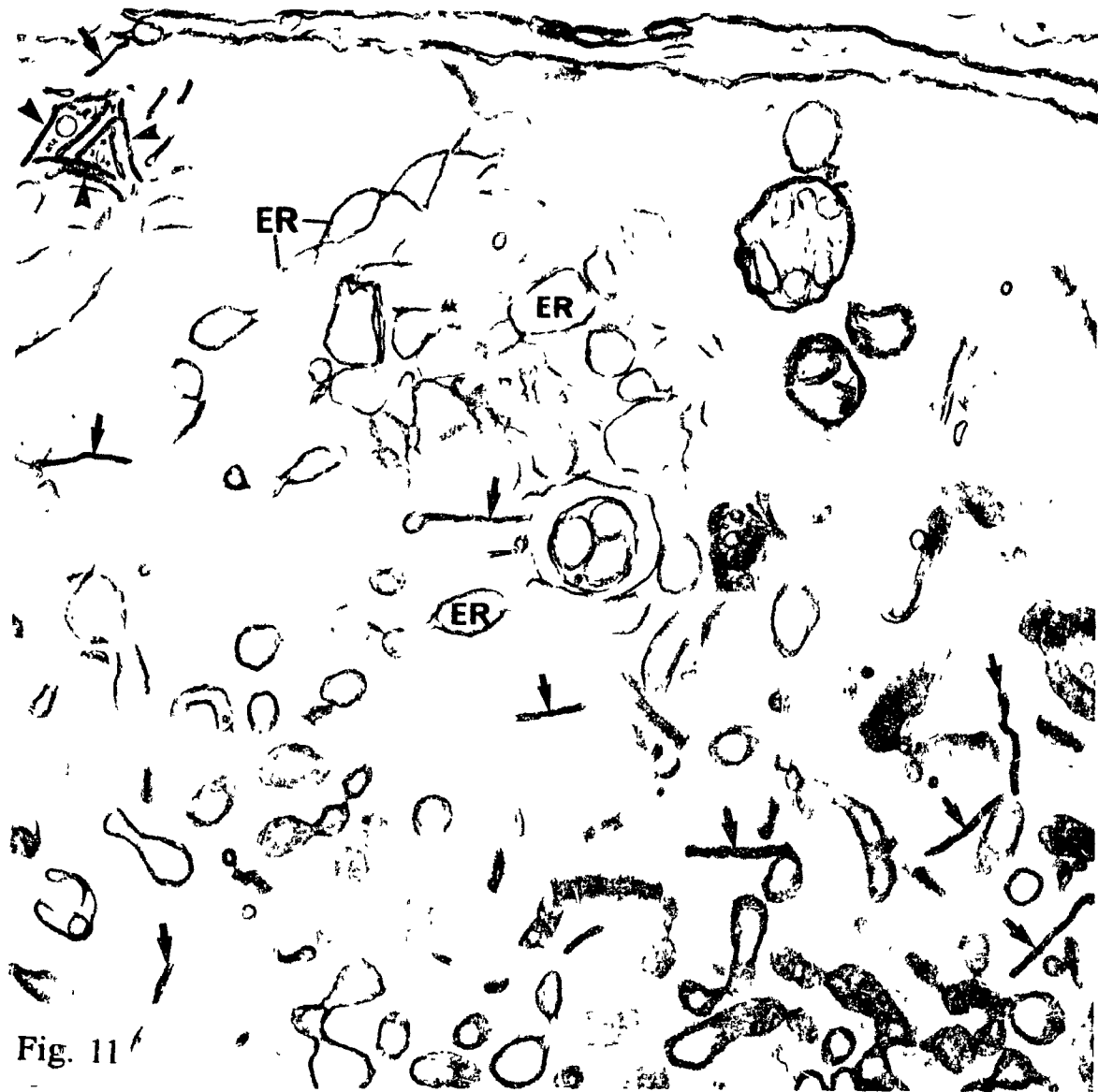


Fig. 11

Fig. 12. Late step 19 spermatids in the lumen (Lu) of the seminiferous tubule at stage VIII of the cycle of the seminiferous epithelium prior to spermiation. These spermatids are still closely associated with cytoplasmic processes of Sertoli cells (S). Cytoplasmic droplets of these spermatids appear at the level of the connecting piece (CP) or neck region of their tails. The droplets contain many flattened saccular elements (arrows) which are loosely arranged or closely stacked, they contain only the occasional small vesicular profile (v) and a few spherical bodies with a double membrane (arrowheads). The plasma membrane in the region of the droplet shows several invagination or pits (P). N nucleus, A acrosomic system of the spermatid head, NE nuclear envelope x 27,000



Fig. 12

Fig. 13. Electron micrograph of crude supernatant of epididymal spermatozoa homogenate from initial low speed centrifugation (1,500 g) In the supernatant fraction, numerous cytoplasmic droplets (CD) are found In addition to saccular elements (arrowheads), many of the droplets contain an electron-dense matrix, circumscribed by their plasma membrane (pl) This fraction was then further fractionated at 200,000 g on a discontinuous sucrose gradient x 17,500

Fig. 14. Electron micrograph of the crude pellet of epididymal spermatozoa homogenate from initial 1,500 g centrifugation Various structures of the spermatozoa predominate with occasional findings of some cytoplasmic droplets (CD) ST sperm tail, N nuclei, P perforatorium x 20,000

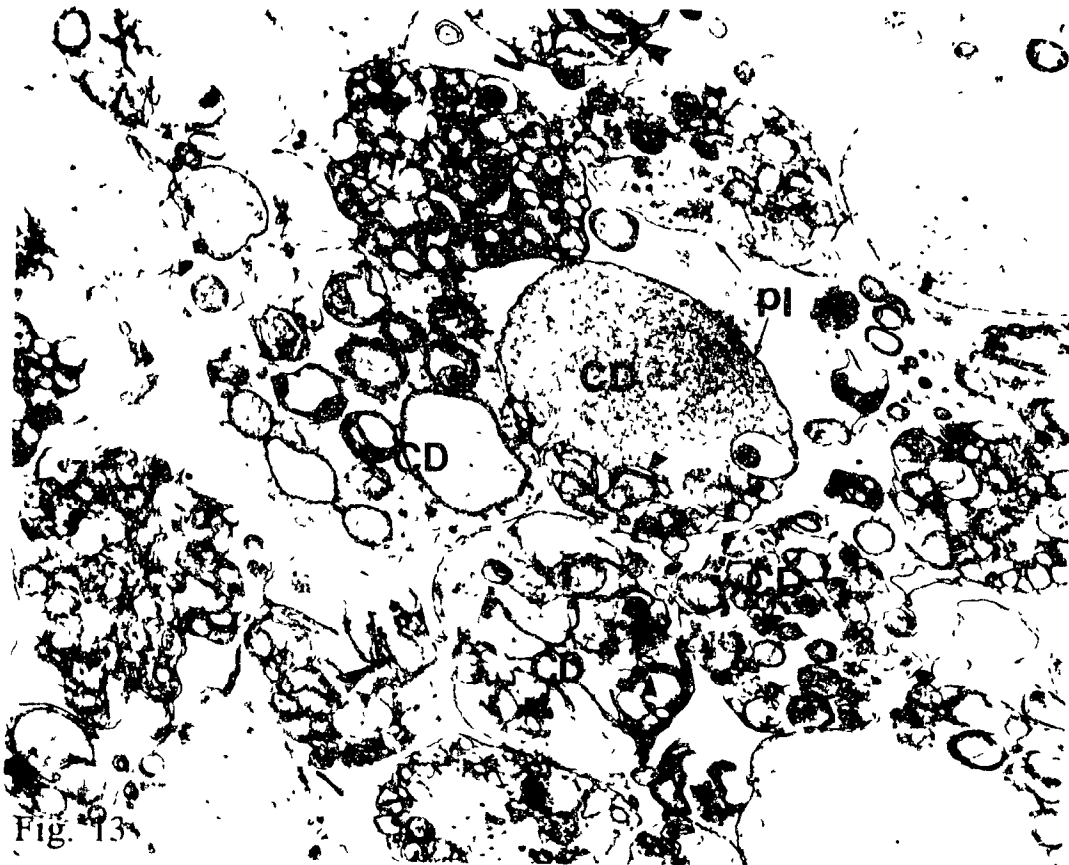


Fig. 13



Fig. 14

Fig. 15. Electron micrograph of fraction 3 (0.8/1.0 M STK buffer) showing numerous flattened, straight (arrows) or C-shaped (arrowheads) saccular elements, enclosed within a remnant plasma membrane (PM). Ag agarose embedding material, arrowheads spherical bodies with a double membrane, v vesicular profiles x 32,500. Inset: Isolated Golgi apparatus from the epithelial principal cells from pellet of the original sperm homogenate. Note that the saccules (S) of this Golgi apparatus are tightly packed, of larger size and associated with numerous small vesicles (v) and tubules (T). This appearance is not found with the saccular elements of the cytoplasmic dioplets x 29,000.



Fig. 5

Fig. 16. Electron micrograph of fraction 4 (0.6/0.8 M STK buffer) showing saccular elements (arrowheads) with similar morphological appearance as in fraction 3. In contrast to saccular elements in fraction 3 where they are enclosed within a plasma membrane in a more discrete fashion, here they are freely and more loosely arranged throughout the fraction. $\times 32,500$

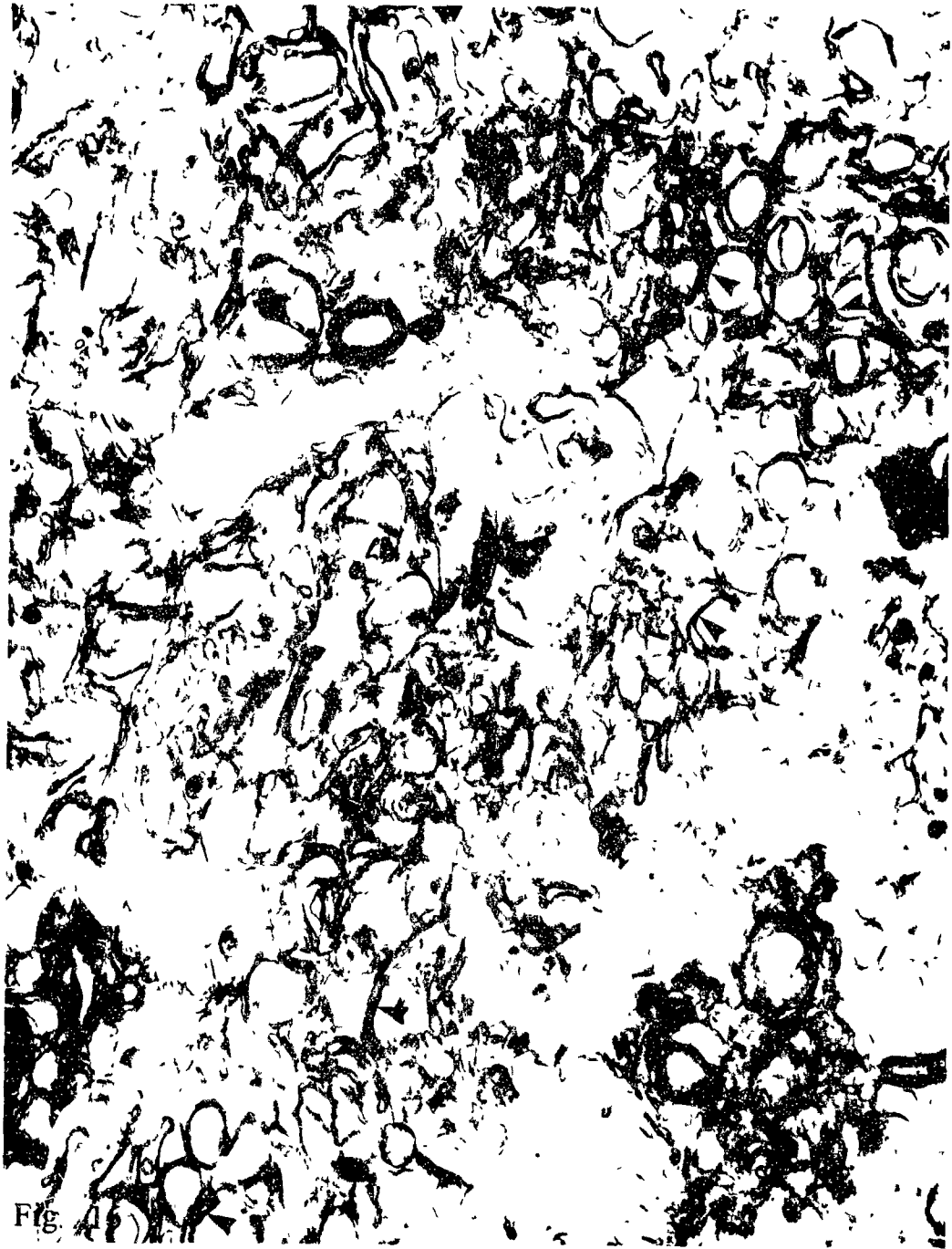


Fig. 1

Fig. 17. Polypeptide compositions of various isolated fractions on a Coomassie Brilliant Blue-stained 8-18% linear gradient SDS-PAGE. All fractions were isolated from spermatozoa from the initial segment and caput epididymis. Major polypeptides observed on fraction S, which represents a crude extract of cytoplasmic droplets, are of molecular masses 21, 41, 44.5, 68, 75, 80 kD. In fraction 3 (lane 3), seven major peptide bands were observed at molecular masses 33, 36.3, 44.5, 68, 75, 97, 110 kD. Six of these polypeptides were more prominent than in the original crude extract (fraction S), thus representing an enrichment of these polypeptides. Note also the similarity of polypeptide composition of fraction 3 (lane 3) and 4 (lane 4) P. Crude pellet (fraction P of Table 1) of the initial centrifugation of spermatozoa homogenate S. Supernatant (fraction S of Table 1) of initial centrifugation. Lane 1 to R fraction 1 to R, respectively, of the subcellular fractionation discontinuous gradient on which fraction S was overlaid, as described in Table 2. Approximately 100 ug was loaded onto each lane except the last lane (lane S) which was loaded with 50 ug of fraction S. Molecular mass of standard polypeptides are denoted by number x 1000.

Fig. 18. Western blots immunostained with anti-sera (anti-SE) raised against fraction 3 isolated from spermatozoa of the entire epididymis. Lane H and I Coomassie Brilliant Blue-stained blots showing numerous polypeptide bands transferred from SDS-PAGE of fraction 3 isolated from caput (lane H) and cauda (lane T) epididymal spermatozoa. Prominent peptide bands on lane H are comparable to those observed on SDS-PAGE (lane 3 of Fig. 17). Lane H3 and T3 similar to lane H and T respectively and immunostained with anti-SE. Note the differences in the immunostaining patterns on caput (H3) and cauda (T3) fraction 3. Polypeptides at 17, 36 and 44.5 kD from caput fraction 3 (H3) were prominently recognized by the anti-SE sera while immunoreaction was most intense between 30 to 70 kD on cauda fraction 3 (T3). Molecular mass of standard polypeptides are denoted by number x 1000.

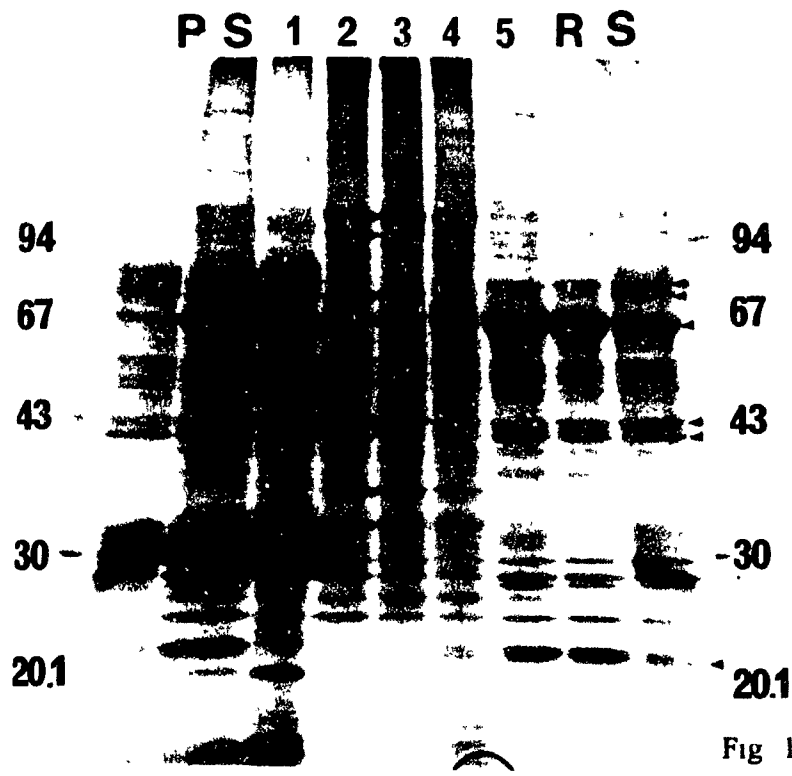


Fig 17

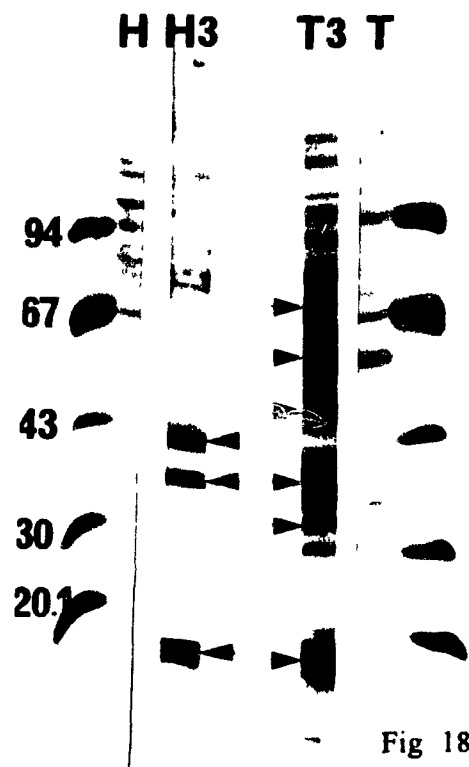


Fig 18

Table 1. Spermatozoa of the initial segment and caput epididymidis fractionated into a pellet (P) and a supernatant (S) fractions. The enzyme activities for the S fraction were as follows: Galactosyltransferase 0.19 ± 0.03 nmols galactose transferred/min/mg protein, Sialyltransferase 0.114 ± 0.02 nmols sialic acid transferred/min/mg protein, Acid phosphatase 68 ± 11.6 nmols PNPP hydrolyzed/min/mg protein.

Percent of total recovered protein or enzyme activities (mean \pm Standard Deviation, n=3)

R S A Relative specific activities are calculated as the percent recovered enzyme activity/percent recovered protein (mean \pm Standard Deviation, n=3)

N D not detectable

Table 1. Distribution of Golgi and lysosomal marker enzymes.

	Fraction P		Fraction S	
	Percent	R S A	Percent	R S A
Protein	74.2±3.5		25.3±3.5	
Galactosyltransferase	58.7±5.9	0.79±0.07	41.3±5.9	1.65±0.25
Sialyltransferase	57.5±7.7	0.78±0.02	42.5±7.7	1.68±0.55
Acid phosphatase	ND	ND	100	3.90±0.50

Table 2. Distribution of recovered enzyme activities of cell fractions. The supernatant fraction isolated from the initial segment and caput epididymal spermatozoa, as described in Table 1, was overlaid on a discontinuous gradient of 0.6, 0.8, 1.0, 1.2 M sucrose in 50 mM Tris HCl, pH 7.4, 25 mM KCl with aprotinin and PMSF as protease inhibitors. Each interface of the gradient from bottom to top of the tube was collected (fractions 1-5 with fraction 1 the resuspended pellet) with the remainder of the material collected and designated fraction R. Aliquots of the fractions were then evaluated for the indicated enzyme activities and protein content. The absolute values of layer 3 were protein, 274 ± 81.8 ug, galactosyltransferase, 3.52 ± 0.474 nmol/min/mg protein, sialyltransferase 1.357 ± 0.146 nmol/min/mg protein, acid phosphatase 396 ± 73.6 nmol Pi/min/mg protein. The results have been averaged from three fractionations ($n=3$) \pm standard error.

Table 3. Distribution of recovered enzyme activities of cell fractions from cauda epididymidis. Fractionation was carried out in a similar way as in Table 2. The absolute values of layer 3 were protein, 511 ± 134 ug, galactosyl-transferase, 0.285 ± 0.0779 nmol/min/mg protein, sialyltransferase 0.0877 ± 0.0213 nmol/min/mg protein, acid phosphatase 324 ± 53.9 nmol/min/mg protein. The results have been averaged from three fractionations ($n=3$) \pm standard error.

Table 2. Distribution of recovered enzyme activities of cell fractions isolated from caput epididymal spermatozoa.

Fraction	Protein (%)	Galactosyl-transferase (%)	Sialyl-transferase (%)	Acid phosphatase (%)
Layer 1	32.8 ± 9.57	9.57 ± 3.77	16.0 ± 7.15	32.0 ± 2.78
Layer 2	7.08 ± 2.24	6.66 ± 1.32	2.46 ± 1.07	8.67 ± 1.82
Layer 3	5.27 ± 1.57	30.6 ± 4.03	20.0 ± 2.15	9.68 ± 3.02
Layer 4	3.95 ± 0.76	12.6 ± 3.89	14.8 ± 1.41	8.99 ± 2.60
Layer 5	7.79 ± 2.73	8.59 ± 0.65	9.65 ± 4.16	5.50 ± 2.49
Layer R	43.1 ± 7.2	31.9 ± 5.98	37.0 ± 1.65	35.2 ± 10.5

Table 3. Distribution of recovered enzyme activities of cell fractions isolated from cauda epididymal spermatozoa.

Fraction	Protein (%)	Galactosyl-transferase (%)	Sialyl-transferase (%)	Acid phosphatase (%)
Layer 1	31.0 ± 7.94	38.8 ± 15.2	25.7 ± 13.3	49.8 ± 9.83
Layer 2	7.22 ± 2.50	7.07 ± 0.762	5.11 ± 1.37	9.80 ± 4.54
Layer 3	6.57 ± 1.72	9.65 ± 1.71	9.63 ± 1.87	13.5 ± 5.32
Layer 4	3.63 ± 0.365	6.10 ± 0.360	4.43 ± 2.02	3.26 ± 1.63
Layer 5	6.09 ± 3.19	3.68 ± 0.635	12.6 ± 7.78	2.00 ± 0.92
Layer R	45.1 ± 18.6	34.8 ± 4.00	42.6 ± 14.3	21.6 ± 11.2

Fig. 19. Cell fractions from the initial segment and caput epididymidis. The results on Table 2 are presented as a modified de Duve plot describing the mean ($n=3$) of three fractionations with relative enrichments of enzyme activity in the fractions of the gradients plotted as a function of the relative protein content recovered for each fraction of the gradient. Significant fold enrichment in galactosyltransferase and sialyltransferase activities are observed in fractions 3 and 4.

CAPUT

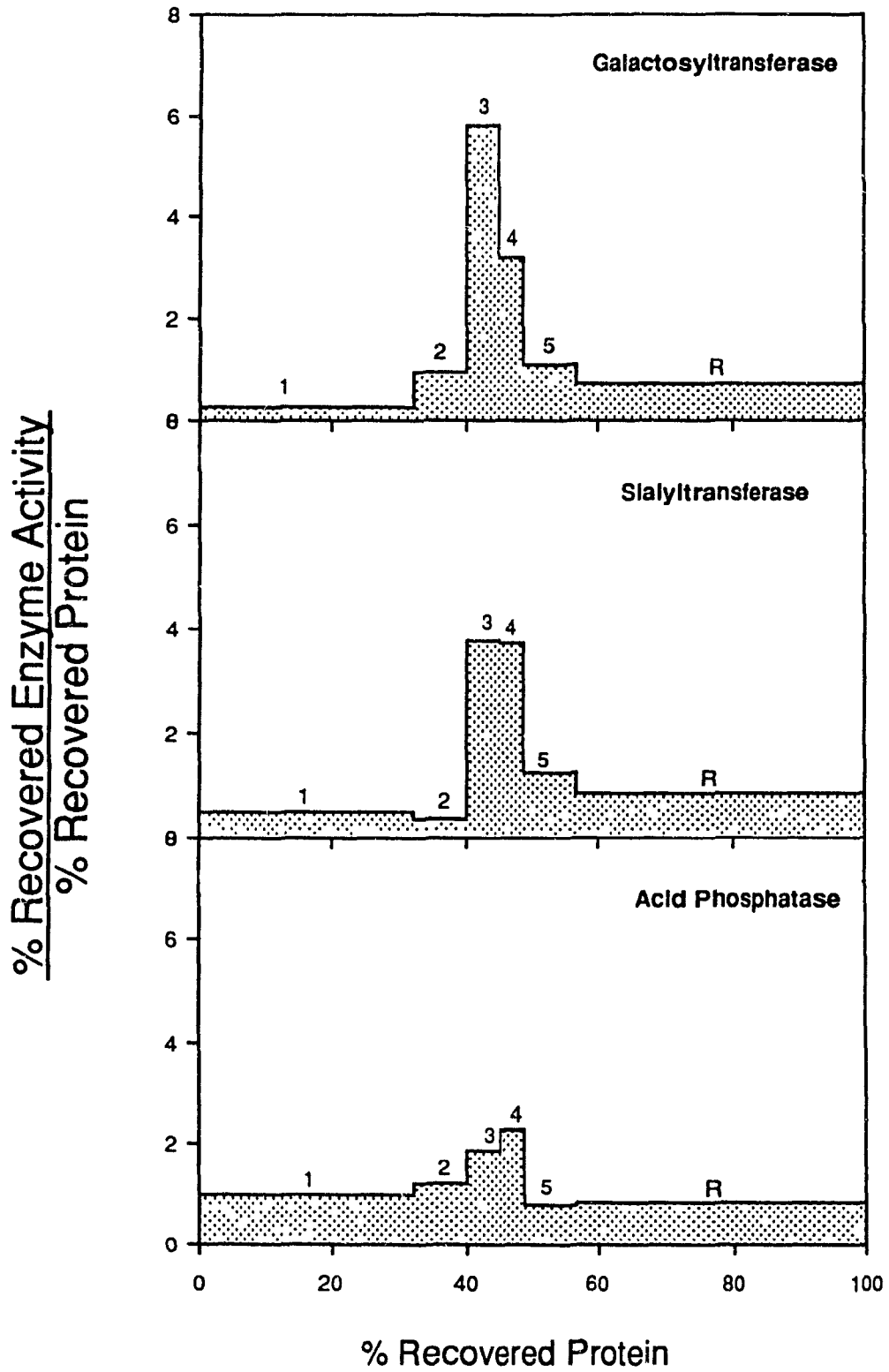


Fig. 20. Cell fractions from cauda epididymidis. The results on Table 3 are presented as a modified de Duve plot describing the mean ($n=3$) of three fractionations with relative enrichments of enzyme activity in the fractions of the gradients plotted as a function of the relative protein content recovered for each fraction of the gradient. Significant enrichment of enzyme activities are not observed in any fractions.

CAUDA

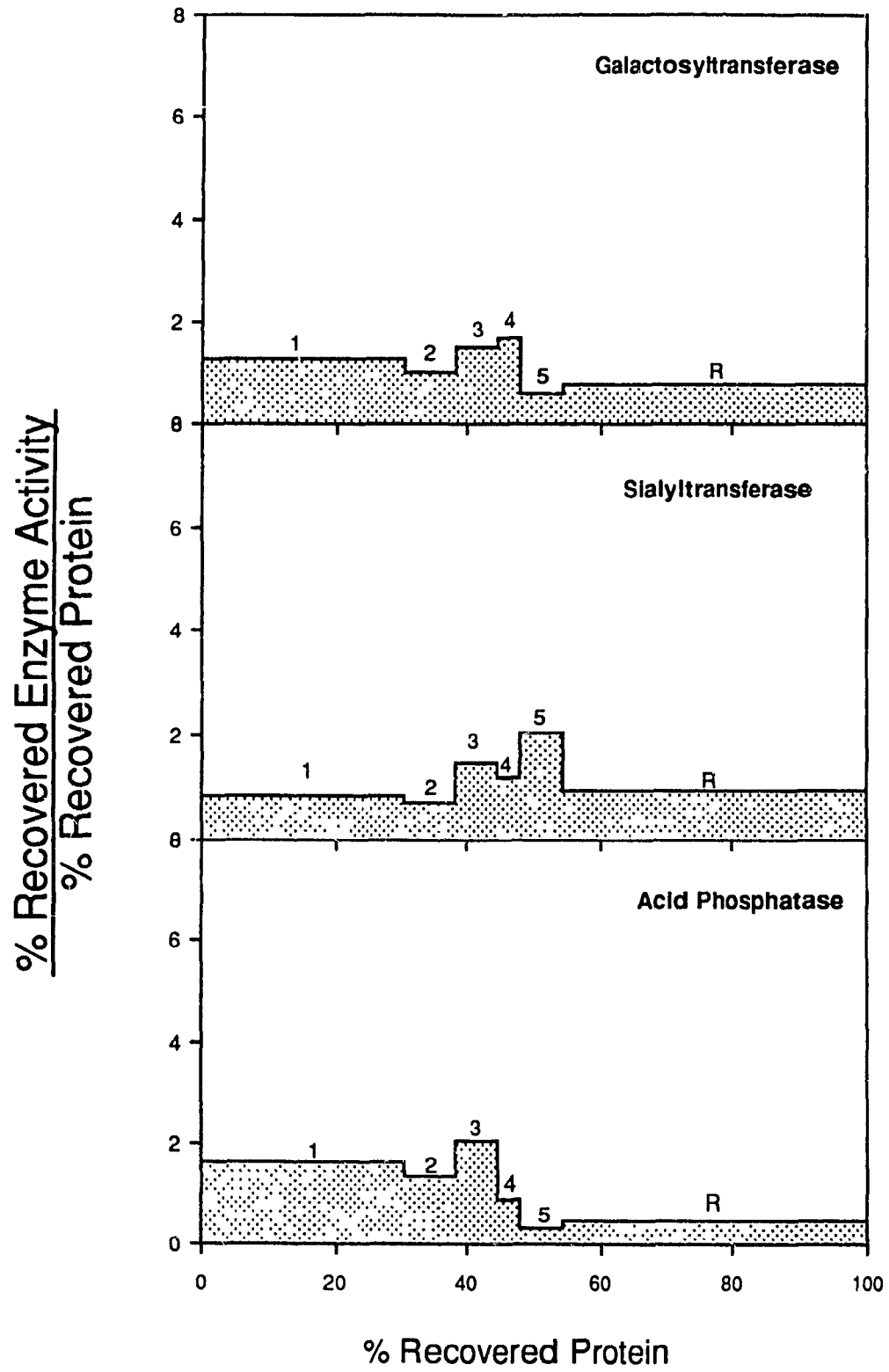


Fig. 21. Effect of trypsinization on galactosyltransferase activity of isolated saccular elements in fraction 3 from initial segment and caput epididymal spermatozoa cytoplasmic droplets. The variations in percentage enzyme activity at exponentially increasing levels of trypsin in the incubation medium are shown in the presence (dotted line) or absence (solid line) of the detergent Triton X-100. Enzyme activity in medium free of trypsin represents 100%. Note that in the presence of detergent, galactosyltransferase activity declines to below 5% at 1 ug/ml of trypsin while in the absence of detergent, the enzyme activity maintains around 60% even at trypsin level of 100 ug/ml.

Table 4. Effect of trypsinization on galactosyltransferase activity in the presence or absence of Triton X-100, as represented on Fig 21. Each value is reported as the mean (n=3) of three experiments within one standard deviation.

Fig. 21. Effect of trypsinization on galactosyltransferase activity

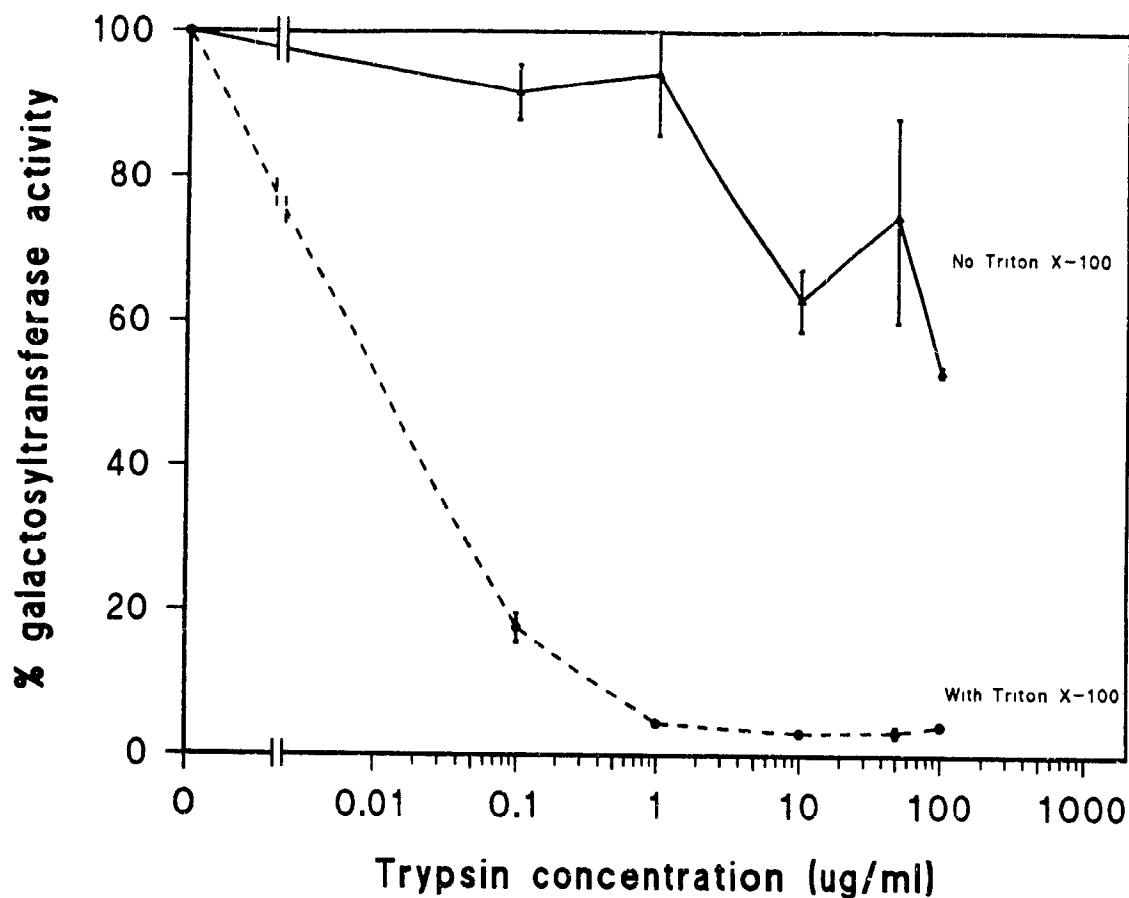


Table 4. Effect of trypsinization on galactosyltransferase activity in the presence or absence of Triton X-100.

Concentration of trypsin ($\mu\text{g/ml}$)	0.1	1.0	10	50	100
% Enz act	91.7 \pm 3.73	94.2 \pm 8.39	63.0 \pm 4.28	74.6 \pm 14.6	53.2 \pm 6.87
% Enz act with Triton X-100	17.7 \pm 1.96	4.47 \pm 2.68	3.07 \pm 4.26	3.24 \pm 7.45	4.11 \pm 1.67

Fig. 22. Low power light micrograph of several tubules of the initial segment of the epididymis immunostained with anti-sera against TGN-38 (dilution 1:20). There is an intense immunoperoxidase reaction (arrows) in the form of a beaded chain overlying the supranuclear region of the epithelial principal cells. Immunoreactivity (arrowheads) also appears over spermatozoa in the lumen (Lu) in the form of small discrete dark dots. IT: intertubular space. x 100.

Fig. 23. High power light micrograph of adjacent tubules of the initial segment of the epididymis immunostained with anti-TGN-38 antibody. Intense immunoperoxidase immunoreactivity (arrows) appears over the Golgi apparatus of the principal cells. An intense reaction (arrowheads) is also evident over the cytoplasmic droplets of spermatozoa in the lumen (Lu), while the remainder of the tails of the spermatozoa are unreactive. IT: intertubular space, Mv: microvilli, n: nuclei of principal cells. x 600.

Fig. 24. Localization of galactosyltransferase in epididymis. High power light micrograph of the initial segment of the epididymis immunostained with anti-sera against β 1,4-galactosyltransferase (1:50 dilution). The Golgi apparatus of principal cells is intensely reactive (G) as are cytoplasmic droplets (arrowheads) of spermatozoa in the lumen (Lu). n: nuclei. x 600.

Fig. 25. Localization of galactosyltransferase in cytoplasmic droplets. On further magnification, it was evident that the immunoreactions are predominantly localized to one pole of the cytoplasmic droplet (arrowheads), coinciding with the location of the aggregated saccular elements. x 1000.



Fig. 22

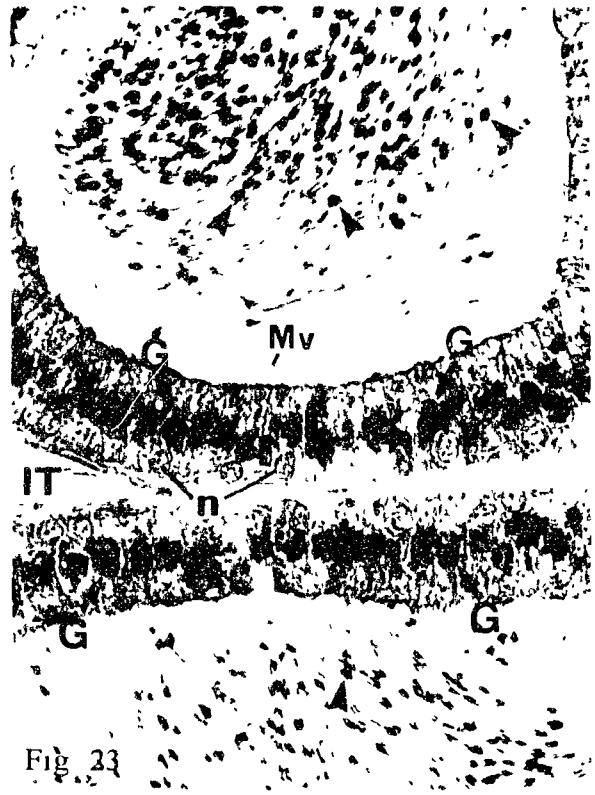


Fig. 23



Fig. 24

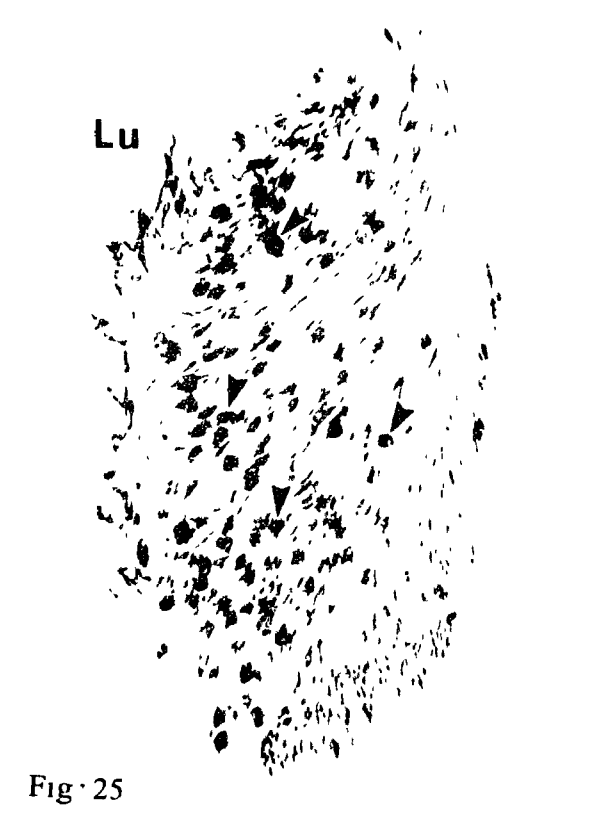


Fig. 25

Fig. 26. Localization of sialyltransferase in epididymis. High power light micrograph of the initial segment of the epididymis immunostained with affinity-purified antibodies against α 2,6- sialyltransferase (1:10 dilution). The Golgi apparatus of principal cells is intensely reactive (G) as are cytoplasmic droplets (arrowheads) of spermatozoa in the lumen (Lu) x 600

Fig. 27. Light micrograph of the corpus epididymis immunostained with anti-sera against β 1,4-galactosyltransferase (1:50 dilution). In contrast to the initial segment in Fig 24, immunoreaction to cytoplasmic droplets (arrowheads) in the lumen (Lu) of the corpus epididymis was much weaker, although reaction to the Golgi apparatus (arrows) of the epithelial principal cells (p) remains comparably intense x 600

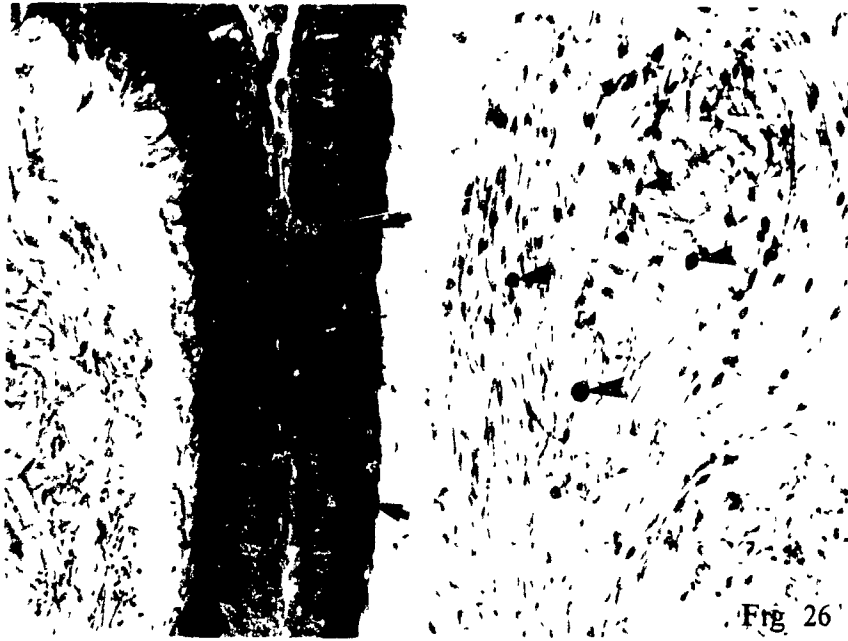


Fig. 26



Fig. 27

Fig. 28. Lowicryl EM immunolocalization of 10-nm gold-decorated secondary antibodies to primary antibodies against β 1,4- galactosyltransferase (1:50 dilution) within droplets of the caput epididymidis. Gold particles (arrowheads) are situated over the saccular elements (S). There is no labeling over the matrix of the droplets, the plasma membrane (Pm) of the droplet or sperm head. A axoneme, AC acrosome, N nucleus of the sperm head, ODF outer dense fibers, P perforatorium, m mitochondrial sheath, Lu lumen of the epididymis \times 40,000

Fig. 29. Lowicryl EM immunolocalization of 10-nm gold-decorated secondary antibodies to primary antibodies against TGN-38 (1:2 dilution) within droplets of the caput epididymidis. Gold particles (arrowheads) are situated over the saccular elements (S). There is no labeling over the matrix of the droplets, the plasma membrane (Pm) of the droplet or sperm head. A axoneme, ODF outer dense fibers, m mitochondrial sheath, Lu lumen of the epididymis \times 40,000

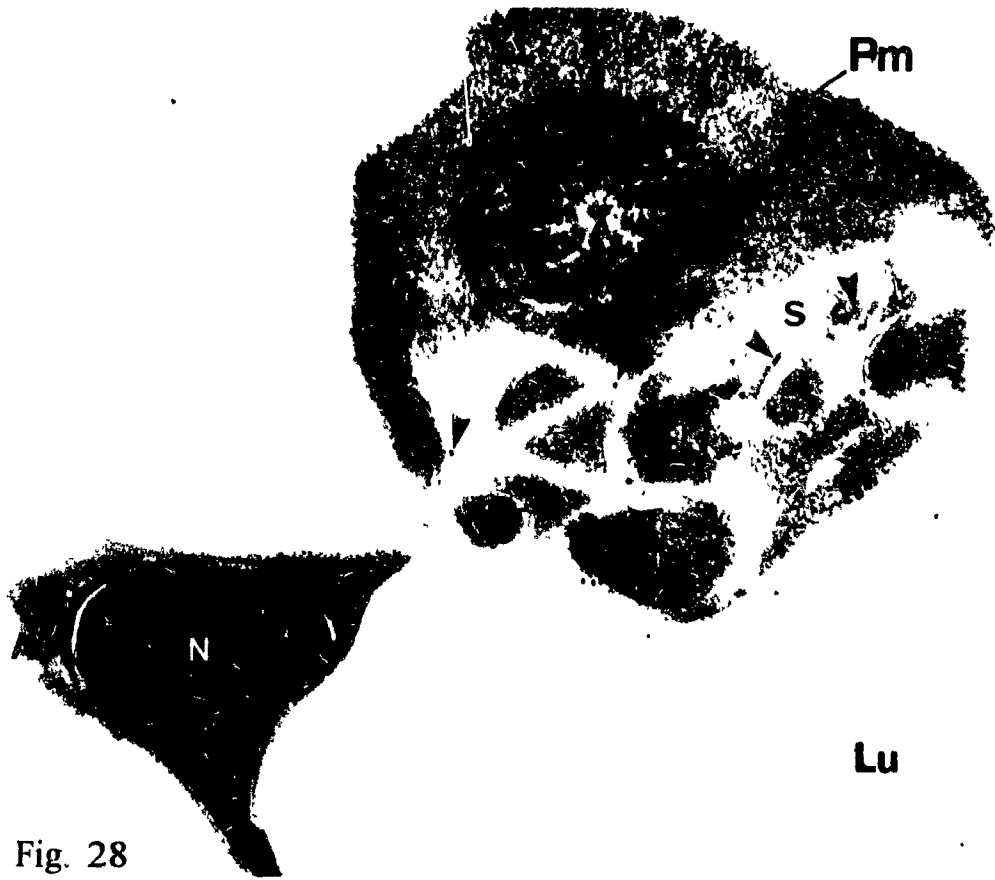


Fig. 28



Fig. 29

Fig. 30. Lowicryl EM immunolocalization of 10-nm gold-decorated secondary antibodies to primary antibodies against α 2,6- sialyltransferase (1:5 dilution) within droplets of the caput epididymidis. Gold particles (arrowheads) are situated over the saccular elements (S). There is no labeling over the matrix of the droplets, the plasma membrane (P) of the droplet or sperm head. A axoneme, ODF outer dense fibers, m mitochondrial sheath x 40,000

Fig. 31. EM of an ultra-thin frozen section obtained from fraction 3 of caput epididymal spermatozoa cytoplasmic droplets immunolocalized with α 2,6 sialyltransferase. The 12-nm immunogold particles (arrowheads) are specifically found over the pale striations corresponding to the saccular elements (S) x 30,000

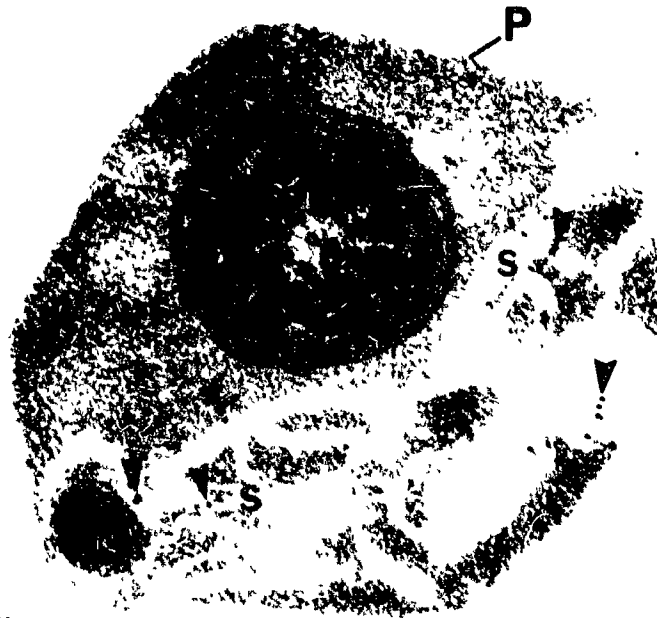


Fig. 30



Fig 31

5

Fig. 32. EM of an ultra-thin frozen section obtained from fraction 3 of caput epididymal spermatozoa cytoplasmic droplets immunolocalized with anti-sera against β 1,4 galactosyltransferase showing abundant gold particles (arrowheads) on saccular elements (S) x 48,000

Fig. 33. On ultra-thin frozen section obtained from cauda epididymal spermatozoa cytoplasmic droplets, immunoreaction (arrowheads) with β 1,4 galactosyltransferase was significantly weaker S saccular elements x 48,000



Fig. 32



Fig. 33

Fig. 34. Low power light micrograph of several tubules of the initial segment of the epididymis immunostained with anti-sera raised against fraction 3 (anti-SE). Immunoperoxidase reaction product is present over spermatozoa in the lumen in the form of discrete dark dots (arrowheads). Immunoreactivity (arrows) also appears over the supranuclear region of the epithelial principal cells x 100

Fig. 35. High power light micrograph of adjacent tubules of the initial segment of the epididymis immunostained with anti-SE antibodies. An intense immunoperoxidase immunoreactivity (arrowheads) is evident over the cytoplasmic droplets of spermatozoa in the lumen, while the remainder of the tails of the spermatozoa are unreactive. Intense immunoperoxidase immunoreactivity also appears over the Golgi apparatus (G) of the principal cells and nuclei of the principal cells x 400

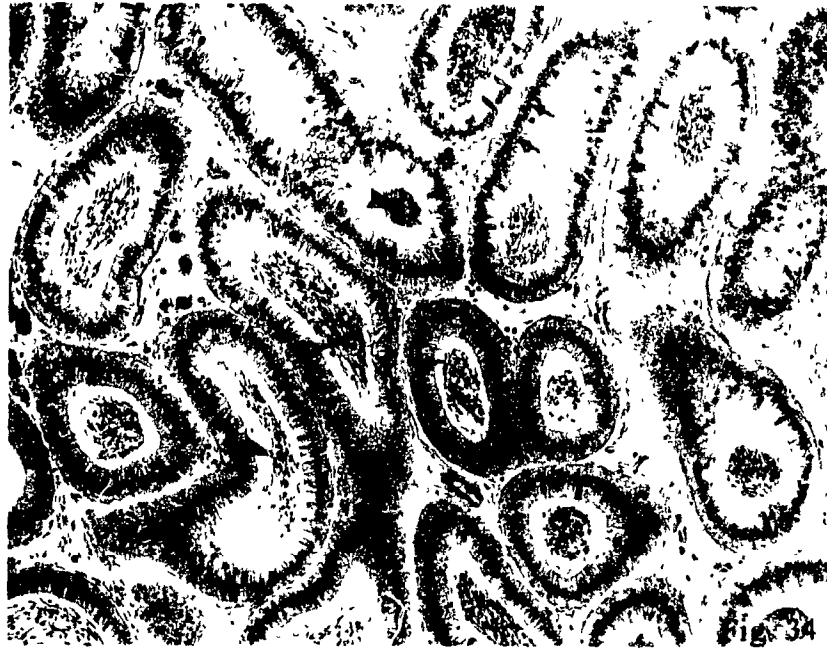


Fig. 36. EM of a Lowicryl section of a cytoplasmic droplet in situ immunolabeled with anti-SE antibodies. Gold particles (arrowheads) decorate the saccular elements (S) occupying one pole of the droplet. ODF: outer dense fibers, m: mitochondria sheath x 45,000

Fig. 37. EM of an ultra-thin frozen section obtained from fraction 3 immunolabeled with anti-SE. The 12-nm immunogold particles (arrowheads) labeled over the pale striations corresponding to the saccular elements (S) x 30,000

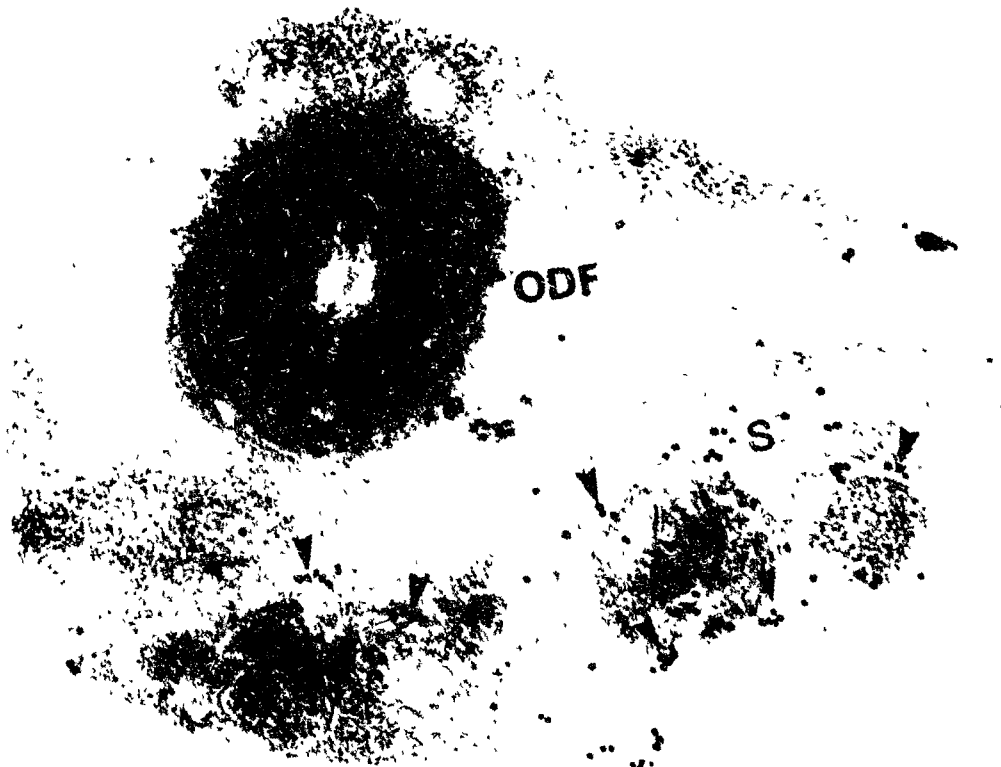


Fig. 36



Fig. 37

Fig. 38. Lectin labeling of cytoplasmic droplets within the caput epididymidis. Ricinus Communis Agglutinin I (RCA-I) colloidal gold (15nm) decorated section of the droplet shows numerous gold particles (arrowheads) over the saccular elements (S) occupying one pole of the droplet indicating the presence of D-galactose containing glyco-conjugates. Label is also seen over the plasma membrane (arrows). A, axoneme, ODF, outer dense fibers, m, mitochondrial sheath x 45,000

Fig. 39. Lectin labeling of a cytoplasmic droplet with Helix Pomatia lectin (HPL) colloidal gold complexes. The majority of gold particles (arrowheads) are present over the saccular elements (S) indicating the presence of N-acetyl galactosamine containing glyco-conjugates. Gold particles are also seen over the plasma membrane (arrow). A, axoneme, ODF, outer dense fibers, m, mitochondrial sheath x 45,000

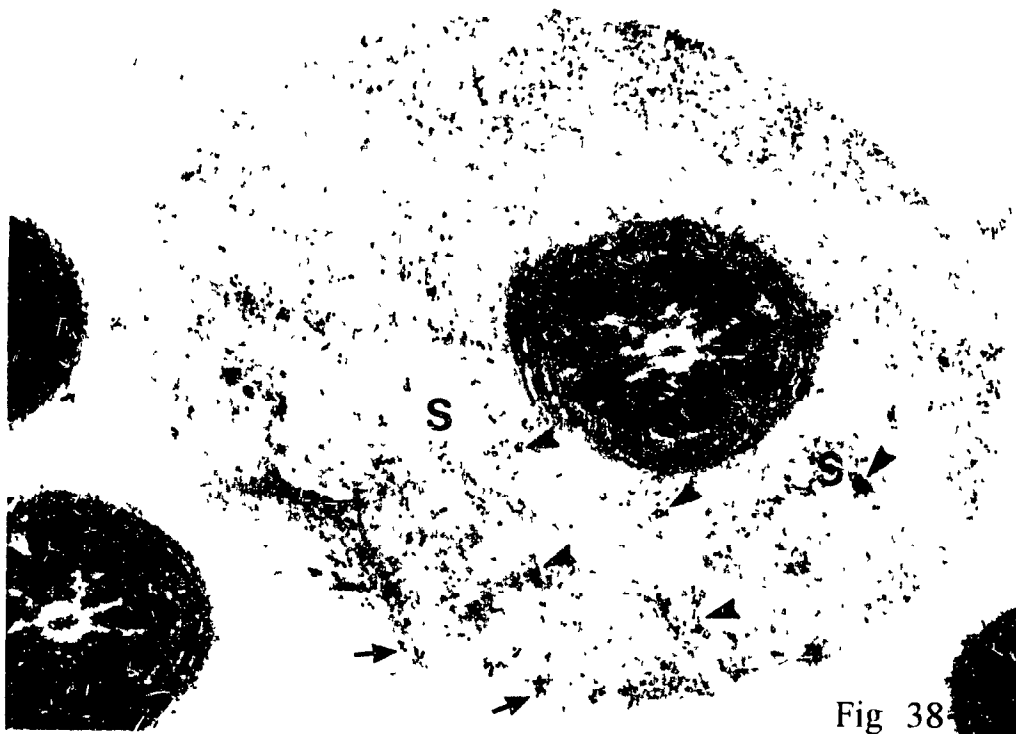


Fig 38

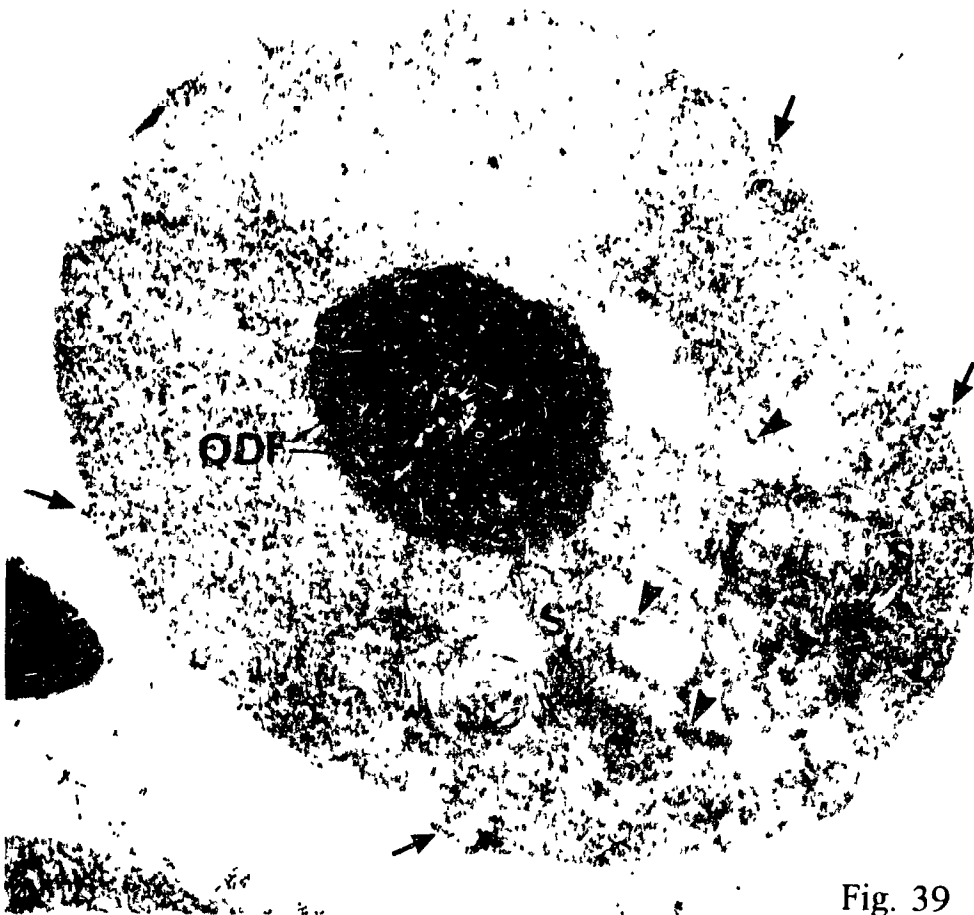


Fig. 39

Fig. 40. SDS-PAGE of polypeptides of isolated cytoplasmic droplets from the initial segment and caput epididymis (lane 1) and endogenous substrates for glycosylation with UDP-[³H] galactose (lane 2) and CMP-[³H] sialic acid (lane 3). Major polypeptides stained by Coomassie brilliant blue (C) were observed at 12, 44.5, 68, and 110 kD (upward pointing arrows), identical to those in Fig. 17. The major sialoprotein acceptors (SA) were observed at 62, 34.5, 20.4, and 14.4 kD (horizontal arrows). Galactose-labeled polypeptides (Gal) were observed between 29 and 280 kD. Molecular mass markers are indicated on the left. Approximately 100 µg protein was loaded on each of lanes 1-3. The exposure time for the x-ray film (lanes 2 and 3) was 1 month.

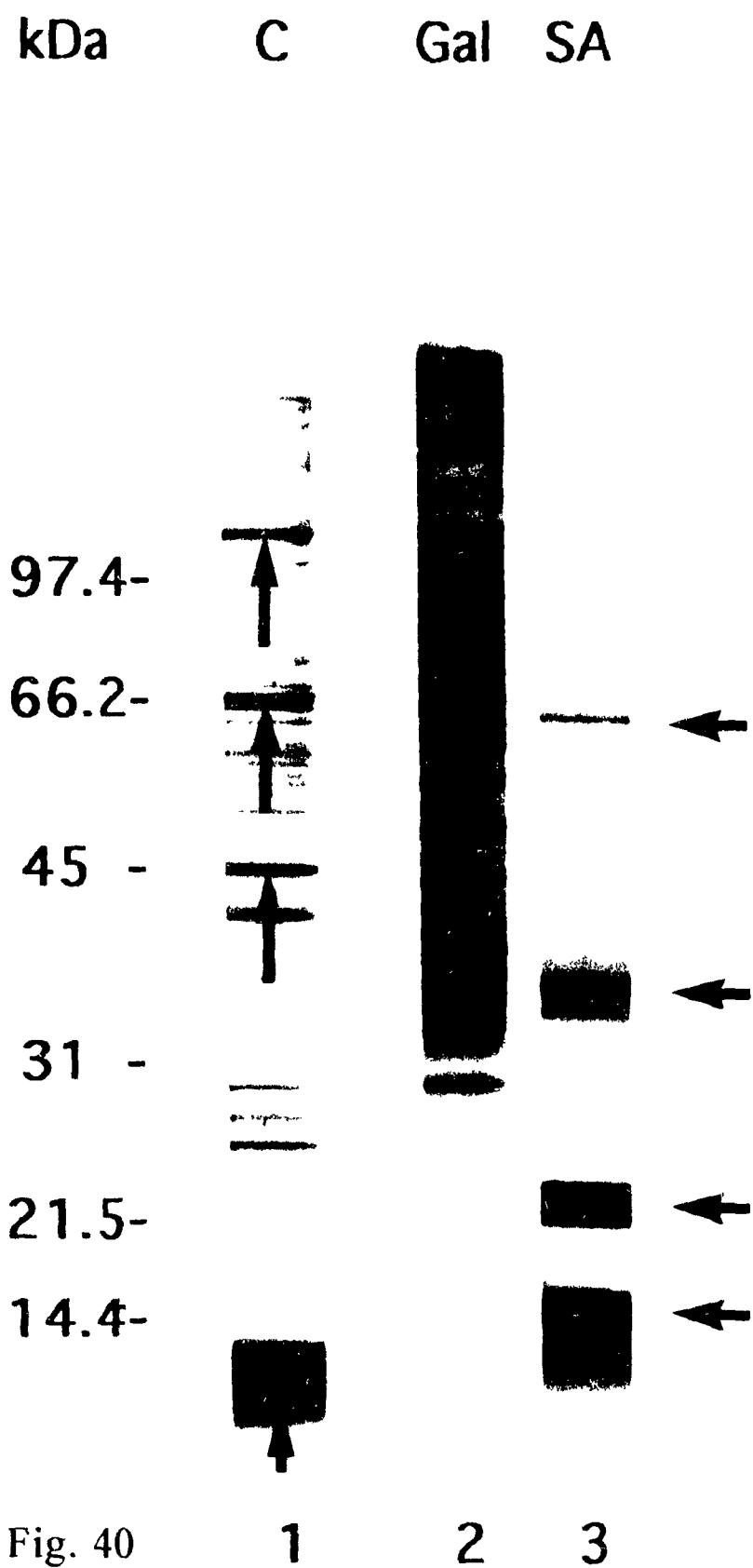


Fig. 40

Fig. 41. Diagrammatic representation illustrating the Golgi apparatus of early spermatids, its fate in late spermatids and presence in cytoplasmic droplets. In early steps of spermiogenesis (steps 1-8), the Golgi apparatus (G) is hemispherical, formed of stacks of saccules and is found next to the acrosomic system (AS) overlying the nucleus (N). In steps 9-16, the Golgi apparatus becomes spherical and occupies the cytoplasmic lobe (CL) of the spermatid. In steps 17-18, the Golgi apparatus is no longer visible as a discrete entity, but many randomly dispersed saccular elements (S) occupy the cytoplasmic lobe. At step 19 of spermiogenesis, the saccular elements segregate themselves at the site of the cytoplasmic droplet (CD), while the other organelles (unused mitochondria, endoplasmic reticulum, lipid bodies, etc.) collectively become aggregated within the residual body (RB). The latter is eventually phagocytosed by the Sertoli cell where it is degraded. The cytoplasmic droplets of spermatozoa in the seminiferous tubules of the testis, rete testis (RT), efferent ducts (ED), and initial segment (IS) are positioned at the level of the neck piece of the tail. In the caput epididymidis, the droplet displaces itself along the tail to now occupy a position next to the annular (An) of the tail. In the corpus epididymidis, the droplet is laterally displaced, while in the cauda epididymidis, many droplets are detached from the sperm. The saccular elements occupy one pole of the droplet but in living specimens they can be seen vortexing the circumference of the droplet in close apposition to the plasma membrane. The combined evidence for the morphological and biochemical presence of Golgi markers and lectin staining within the saccular elements of the droplet and modification of endogenous glycoproteins suggest that the saccular elements are modified Golgi/TGN components.

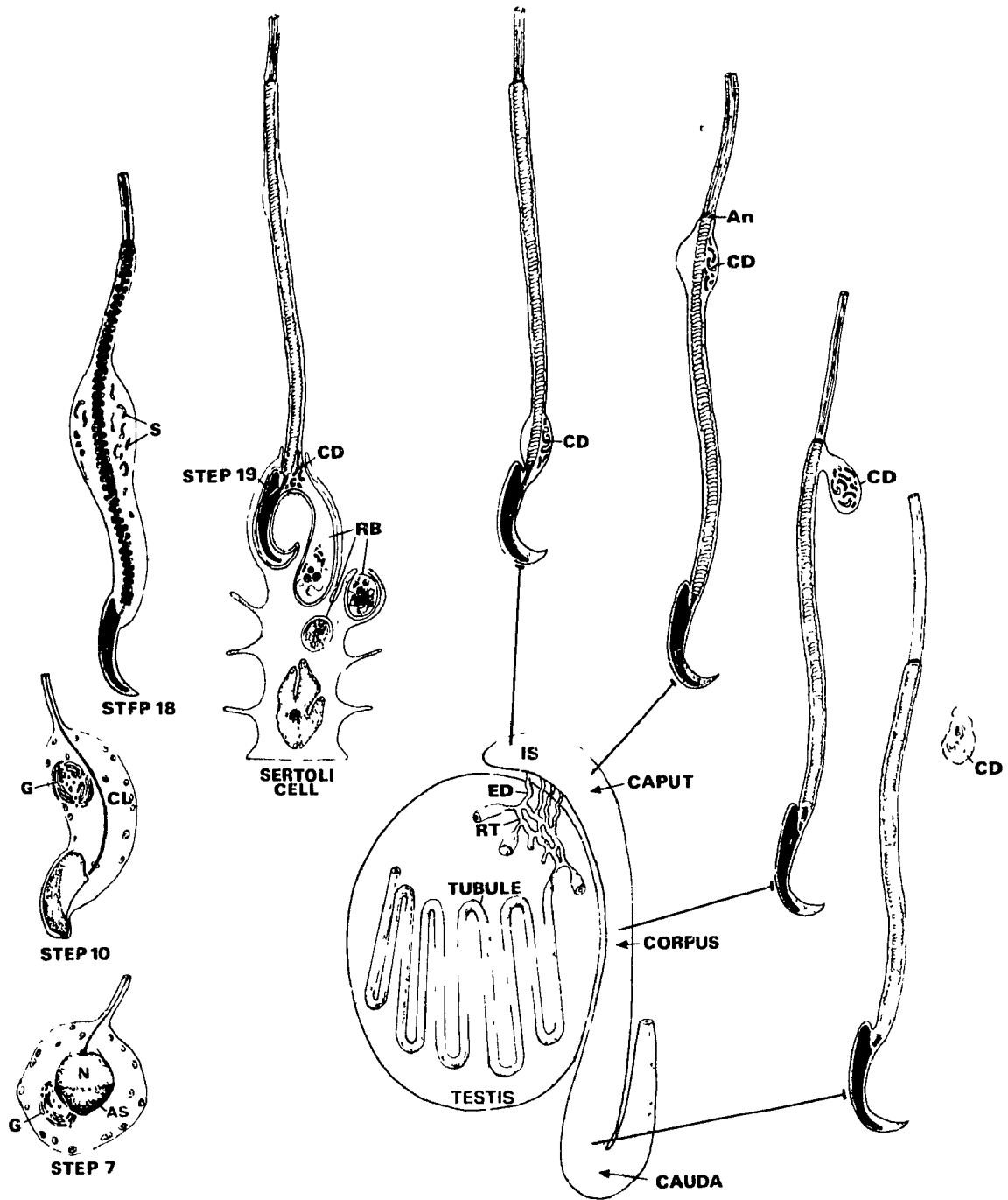


Fig 41

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