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Structure and Function of the Adult Rat Vas Deferens

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Short Title: Structure and Function of Vas Deferens

To my family Dr. Barkev, Alice, and Maria Andonian

ABSTRACT

Epithelial cells lining the adult rat vas deferens show marked structural differences in the proximal, middle, and distal regions of the adult rat vas deferens reflective of diverse functional activities. Their structural features were examined in the electron microscope of glutaraldehyde-fixed, Epon-embedded material. The major epithelial cells were principal and basal with occasional clear and narrow cells in the proximal region. In the middle region, principal cells often contained blebs of their apical cytoplasm which extended far from the cell surface. In the distal region, the infranuclear cytoplasm of principal cells showed whorls of smooth endoplasmic reticulum (sER) with cavities containing membranous structures. In this region, the dilated intercellular spaces between adjacent principal cells and overlying basal cells contained membranous profiles similar to those seen in the cavities of the sER. The lamina propria of the distal region showed vascular channels. Four aspects of the function of the vas deferens were examined by light microscope immunocytochemistry of Bouin-fixed, paraffin-embedded material. First, the expression of aquaporin-1 by endothelial cells of the vascular channels in the lamina propria of the distal region, suggested water transport from the lumen of the vas deferens via the dilated intercellular spaces to underlying vascular channels, the function of which may be to concentrate sperm. Second, the expression of 3β-hydroxysteroid dehydrogenase by principal cells was indicative of steroid synthesis. Third, the ability of the vas deferens to create a special environment for protecting spermatozoa in the lumen was investigated using anti-glutathione S-transferase (GST) antibodies. Both principal and basal cells showed varying degrees of GST expression in the different regions of the cauda and vas deferens, suggesting a complex, changing environment of substrates to which epithelial cells and sperm are subjected. Fourth, the epithelial cells lining the cauda epididymidis and vas deferens were investigated for their secretory and endocytic functions. The expression of different lysosomal enzymes, namely, cathepsins A, D, B, and sulfated glycoprotein (SGP)-1 was not only cell but also region-specific suggesting differences in the type of substrates internalized by these cells. SGP-2, a secretory protein was expressed by principal cells in a manner that suggested its secretion into the lumen where it may function in relation to sperm. The endocytic receptor, low density lipoprotein receptor related protein-

2 (LRP-2) was noted in spherical structures on the apical surface of principal cells indicative of endosomes and suggestive of their role in the uptake of various ligands, including SGP-2, for which it has a high binding affinity. Thus SGP-2 in the cauda and vas deferens is not only secreted but endocytosed by principal cells, suggestive of an active turnover in the lumen. In summary, epithelial cells of the vas deferens appear to be involved in 1) eliminating water from the vas deferens lumen, 2) synthesis and secretion of steroids, 3) providing ample protection of sperm from harmful circulating electrophiles during their storage and 4) expression of a variety of lysosomal proteins, SGP-2 and LRP-2 often in cell and region specific manner. All of these functions must aid in sperm maintenance and protection while they are stored in this region.

RÉSUMÉ

L'épithélium des canaux déférents du rat présente des différences structuralws marquées le long des portions proximales (i.e. adjacentes aux canaux épididymaires), intermédiaires et distales de ces canaux. Deux types de cellules prédominent dans cet épithélium, les cellules prismatiques ou principales et les petites cellules basales. Ainsi par exemple, comme le rélève l'examen à l'aide du microscope électronique, les cellules principales montrent à leur apex, en plus des microvillosités, des processus globulaires que l'on trouve que dans le segment intermédiaire; de plus, dans la portion distale, le cytoplasme des cellules principales montre, dans la région infra-nucléaire, des empillements massifs de citernes du réticulum endoplasmique lisse associés a des vacuoles qui se trouvent à proximité d'espace inter-cellulaires dilatés semblables à ceux observés dans les épithéliums impliqués dans le transport de l'eau (épithélium intestinal, de la vésicule biliaire etc.) Vers les capillaires sous-épithéliaux.

Au cours de la présente étude, quatre caractéristiques immunocytochimiques des canaux déférents ont été examinées sur des coupes de matériel fixé au Bouin et enrobé dans la paraffine. A) L'expression de l'aquaporine-1 a été demontré dans les cellules endothéliales des petits vaisseaux sous-jacents à l'épithélium de la portion distale des canaux déférents, ce qui suggère un rôle de ces dernières cellules dans le transport d'eau de la lumière des canaux vers les vaisseaux sous-jacents. B) L'expression de la 3β-déhydroxystéroïde-déhydrogénase par les cellules épithéliales principales démontre le rôle de ces cellules dans la synthèse des stéroïdes. C) Les cellules principales et basales expriment à des degrés divers le long des canaux déférents la gluthation-S-transférase (GST) un agent électrophile qui protège les cellules (les spermatozoïdes vraisemblablement) des électrons libres. D) L'activité secrétrice et endocytique des cellules principales a été également analysée cytochimiquement à l'aide d'anticorps anti-cathpsine A, D, B (enzymes lysosomiaux), de la glycoprotéine sulfatée-1 (SGP-1) et du récepteur des lipoprotéines de faible densité LRP-2. Nos observations montrent, pour ces diverses substances, une distribution variable main caractéristique le long des canaux déférents. Ces observations diverses soulignent des variations fonctionnelles significatives des cellules épithéliales le long des canaux déférents. En bref, ces cellules paraissent

impliquées: 1) Dans le transport de l'eau et des électrolytes de la lumière des canaux vers le système vasculaire. 2) Dans la synthèse et la secrétion des stéroïdes. 3) Dans la protection des spermatozoïdes des électrons libres par l'intermédiaire de la GST. 4) Dans la biosynthèse d'enzymes lysosomiaux et l'activité endocytique des cellules principales. Ces diverses fonctions doivent contribuer à la protection des spermatozoïdes au cours de leur entreposage dans les canaux déférents.

List of Abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
AQP-1	aquaporin-1
Cauda	cauda epdidymidis
cm	centimeter
DC	distal region of cauda epididymidis
DVD	distal region of the vas deferens
ER	endoplasmic reticulum
Fig.	figure
GST	glutathione S-transferase
K Da	kilodaltons
LRP-2	low density lipoprotein receptor related protein-2
MC	middle region of cauda epididymidis
μm	micrometer
MVBs	multivesicular bodies
MVD	middle region of the vas deferens
MVDP	mouse vas deferens protein
nm	nanometer
PAS	periodic acid Schiff
pH	potential of Hydrogen
PVD	proximal region of the vas deferens
rER	rough endoplasmic reticulum
sER	smooth endoplasmic reticulum
SGP-1	sulfated glycoprotein-1
SGP-2	sulfated glycoprotein-2

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Chapter Two:

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PREFACE

This thesis is a collection of three articles which have been accepted by Journal of Andrology for publication. They are included as in their final accepted form with the exception of the references which are included in chapter six to avoid repetition and to save space. All of the experiments in these articles were performed by the candidate, except for the second article where the candidate received help from Kara Golan. In addition to these articles which comprise chapters two, three and four, this thesis also contains a complete and up-to-date Literature Review as the first chapter that serves as a general introduction to the three articles. Chapter five serves as a final Summary and Conclusion for the thesis.

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CHAPTER ONE:
LITERATURE REVIEW

This literature review chapter is divided into four sections. The first section deals with the gross structure of the rat vas deferens. The second section presents histological appearance of each of the cell types found in the epithelium of the rat vas deferens. The third section discusses the functional implications of the structural features presented in preceding sections. Finally, the fourth section presents the objectives of this thesis.

1.1. Gross Structure of the Vas Deferens:

The adult rat vas deferens is an epithelial lined muscular tube, whose length has been reported to be in the range 4.5-6.0 cm (Flickinger, 1973; Hamilton and Cooper, 1978; Kennedy and Heidger, 1979). It originates as a straight tube from the coiled cauda epididymidis in the scrotum and ends as the ejaculatory duct in the pelvis (Hamilton and Cooper, 1978). While Hamilton (1975) found the junction between cauda epididymidis and vas deferens abrupt, Kennedy and Heidger (1979) found that the transition was gradual. The rat vas deferens has been divided on the basis of luminal diameter, mucosa and blood supply into proximal, middle and distal regions in addition to a terminal portion within the prostate gland (Flickinger, 1973; Hamilton and Cooper, 1978). The precise divisions between these regions appear to be arbitrary and not based on major landmarks. While an ampullary region, where the epithelium is thrown into numerous folds with tubular branches and crypts actively involved in phagocytosing spermatozoa, has been noted in the monkey and human vas deferens (Popovic et al., 1973; Hoffer, 1976; Ramos, 1979), it is not recognized in the rat vas deferens (Flickinger, 1973; Hamilton and Cooper, 1978).

The luminal diameter is greatest in the initial portion of the proximal segment (340-350 μm), where the muscular wall was the thinnest; the diameter decreases to approximately one-half its initial diameter by the point of transition to the distal vas (Kennedy and Heidger, 1979). This is due to the fact that the epithelium lining the entire vas deferens shows an increase in cell height, microvilli, and infranuclear cytoplasm (Hamilton and Cooper, 1978). Another factor contributing to decreased luminal diameter is the fact that in contrast to the circular profile of the mucosa of the proximal region, the mucosa of the distal region is also thrown into extensive infoldings, which appears to be spirally arranged down the length of the tube (Hamilton and Cooper, 1978; Kennedy and Heidger, 1979; Ramos, 1979).

There is a gradual transition from the characteristics of the proximal region to the features of the distal region such that the morphology of the mucosa of the middle region shares features of both proximal and distal regions of the vas deferens. (Flickinger, 1973).

The pseudostratified epithelium of the vas deferens is composed of cuboidal to columnar cells and flattened basal cells, the specific characteristics of each of these cell types will be discussed later. A basement membrane separates the epithelium from the loose lamina propria with blood capillaries (Niemi, 1965). The submucosa is composed of inner circular and outer longitudinal smooth muscle layers which get thicker distally (Hamilton and Cooper, 1978). The muscular wall ranged in thickness from 90 μm in the initial portion of the proximal region to a maximum of 825 μm in the distal region (Kennedy and Heidger, 1979).

In the distal region, capillaries of the lamina propria form a subepithelial network and extend underneath the infoldings. These eventually join to form a complex venous plexus peripheral to the lamina propria. By injecting a bolus of ink into the aorta above the testicular artery, Hamilton and Cooper (1978) found that this plexus is supplied directly by the differential artery and is drained by the differential vein. This venous plexus is continuous with the vascular channels of the corpus spongiosum (Hamilton and Cooper, 1978). It was suggested to act as a large pool for diffusion of materials out of the duct and as an erectile tissue that could act to stiffen the duct during emission phase of ejaculation (Hamilton and Cooper, 1978). The increase in vascularization and complexity of the epithelium in the distal regions was suggested to be due to being more active physiologically (Ramos, 1979). The function of these vascular channels will be discussed further in water transport function of the vas deferens.

The blood supply for the different regions of the vas deferens is different. The proximal vas receives its blood supply from spermatic arteries and is best fixed by retrograde perfusion, whereas the distal vas receives its blood supply though deferential artery and is best fixed by direct perfusion (Hamilton and Cooper, 1978; Kennedy and Heidger, 1979). These investigators recognized the importance of fixation in situ by vascular perfusion of 5% glutaraldehyde in the detection of morphological variations in the muscle wall organization,

luminal configuration, and epithelial height of the normal rat vas deferens. For this reason, in the present study, 5% glutaraldehyde was used in anterograde direction above the bifurcation of the testicular artery to study the structure of the vas deferens.

1.2. Histology of the Vas Deferens:

1.2.1. Principal Cell:

1.2.1a. General Features:

The major columnar epithelial cell type in the vas deferens is the principal cell. The plasma membrane of contiguous cells is highly interlocked and interdigitated and terminal bars are extensive. The infolding of the plasma membrane is prominent between the principal cells and the underlying flattened basal cells (Niemi, 1965). As in the case of other epithelia, the apical plasmalemma covering the microvilli is thicker (10 nm) than that along the lateral and basal cell surfaces (7.5 nm). It lacks the rich coating of filamentous knap or "fuzz" seen along the apical surfaces of gastric and intestinal epithelia (Friend and Farquhar, 1967). On the apical free surface of principal cells, numerous long microvilli (stereocilia) protrudes into the lumen (Flickinger, 1973). These neither anastomose nor branch, but most of them are long and sometimes contain few actin filaments, and the central compartments are more electron dense than the rims (Niemi, 1965). The microvilli of principal cells in the vas deferens are morphologically similar to, but larger in diameter than, those present in the epididymis but differ from those of a brush border of the kidney tubule and bowel by being highly irregular in length and size (Niemi, 1965; Hamilton et al, 1977). The microvilli are PAS-positive indicative of glycoproteins attached to them (Niemi, 1965).

The principal cell cytoplasm contains a large and prominent Golgi apparatus in its supranuclear position (Niemi, 1965; Flickinger, 1973). Each Golgi stack is composed of six to eleven cisternae with an inner, concave surface and an outer, convex surface (Friend and Farquhar, 1967). Other smooth surfaced cisternae usually occur in the cytoplasmic core circumscribed by the Golgi stacks. These have a random orientation and tend to occur singly rather than in stacks (Friend and Farquhar, 1967). The majority of vesicles associated with the cisternae are smooth-surfaced and measure 60 nm in diameter, but 10-20% of those present are of the coated variety and measure 75 nm. No secretory product is discernible in

the vacuoles or cisternae of the Golgi complex (Friend and Farquhar, 1967). Some cisternae of the rough endoplasmic reticulum (rER) are found lateral to the Golgi region. However, the amount of rER declines toward the apical ends of the cells where tubules of the smooth endoplasmic reticulum (sER) become more prominent (Flickinger, 1973).

Friend and Farquhar (1967) noted two types of coated vesicles in the principal cells of the vas deferens. The large, 100 nm or more in diameter ones are seen exclusively near the apical cell surface. The smaller ones, 75 nm are most numerous in the Golgi zone and occasionally can be seen in continuity with Golgi cisternae or with the smooth surfaced cisternae in the Golgi cytoplasmic core. However, individual small coated vesicles can be found distributed throughout the cytoplasm, particularly adjacent to lysosomes and near the apical and lateral cell membranes. The content of the smaller coated vesicles is generally more granular and denser than that of the larger ones (Friend and Farquhar, 1967). The large coated vesicles are formed at the apical cell surface by pinocytic invagination of the apical cell membrane, move toward and fuse with multivesicular bodies (MVBs) and serve to transport absorbed protein from the duct lumen to the lysosomes. The role of these large coated vesicles in endocytosis will be discussed later. The small coated vesicles originate from Golgi cisternae, and move to a peripheral location in the cell. Some of these apparently transport acid hydrolases, from their site of packaging (Golgi apparatus) to their site of action (MVBs). Others apparently fuse with the surface membrane but the nature of their content remains unknown (Friend and Farquhar, 1967). The role of these small coated vesicles in transport of lysosomal enzymes and secretory products will be discussed later.

The apical cytoplasm of principal cells of the rat vas deferens protrudes into the lumen (Niemi, 1965). These bleb-like formations of the apical cytoplasm are also found in the monkey vas deferens (Ramos, 1979). These large bulbous protrusions are devoid of microvilli (Flickinger, 1973). Moreover, the lumen contained membrane bounded spheres of cytoplasm resembling the protrusions. Tubules and some cisternae of sER are prominent in the protrusions and detached spheres in the lumen (Flickinger, 1973). The function of these cytoplasmic blebs in secretion will be discussed later.

The infranuclear region contains linearly arranged ER cisternae, which are mainly

smooth ER (Niemi, 1965). The function of sER in steroid synthesis will be discussed later. The basal cytoplasm is mostly vacuolar which often form intercellular dilations that appeared as vertical clefts (Niemi, 1965). The function of intercellular dilations in water transport will be discussed later. The base of principal cells often contacts the underlying basement membrane by means of only small foot-like processes. Such a poor association of these cells with the basement membrane might, therefore, account for the numerous anchoring fibrils found associated with the basement membrane; these fibrils have been reported to hold the principal cells in place during the vigorous contractions of the smooth muscle coat (Clermont and Hermo, 1985).

1.2.1b. Proximal Region:

Principal cells of the proximal region are low to intermediate in height (8-20 μm) with a short microvillous border (2.5-5 μm) (Kennedy and Heideger, 1979). Invaginations and coated pits are visible on the cell surface between the microvilli (Niemi, 1965; Friend and Farquhar, 1967; Flickinger, 1973). The apical cytoplasm of principal cells in this region is characterized by large numbers of large (100 nm) smooth and coated vesicles and MVBs (Friend and Farquhar, 1967; Flickinger, 1973). A small number of large smooth vesicles, MVBs, and lysosomes are also present in the supranuclear cytoplasm (Flickinger, 1973). The membrane of the large (100 nm) apical vesicles has the same thickness as that of the apical plasma membrane and are involved in transport of absorbed protein from the lumen to MVBs (Friend and Farquhar, 1967). The most commonly encountered MVBs are large and spherical, and occur either singly or in clusters of two or three in the apical cytoplasm. Typically these structures contain relatively few vesicles in a matrix of low density and a clump of finely granulate material. Both the membrane of the MVBs and that of its contained vesicles are of the thicker (10 nm) variety like the apical plasma membrane (Friend and Farquhar, 1967). Round and oval profiles of mitochondria with plate-like cisternae are scattered throughout the cytoplasm (Flickinger, 1973). The presence of large number of mitochondria suggested that high levels of energy produced could be used for the voluminous fluid absorption (Niemi, 1965).

The nuclei of principal cells in the proximal region are ovoid in shape, 7-10 μm in

length, and located in the basal cytoplasm of the cells with their longitudinal axis parallel to the underlying lamina propria (Kennedy and Heideger, 1979). The basal and perinuclear cytoplasm of principal cells in the proximal region contain abundent anastomosing cisternae of rough ER (Flickinger, 1973). Free ribosomes are present between the elements of rER in the basal cytoplasm, but diminishes in number in the apical parts of the principal cell (Flickinger, 1973).

1.2.1c. Middle Region:

The morphology of principal cells of the middle region shares features of both proximal and distal regions of the vas deferens. There is a gradual transition from the characteristics of the proximal region to the features of the distal region (Flickinger, 1973). Principal cells a few millimeters to the proximal side of the middle region of the vas deferens show mainly a cytology similar to the proximal segment, while samples of the middle region contain some small whorls of membranes, suggesting that the cells may progressively acquire larger amounts of sER with increasing distance along the vas deferens (Flickinger, 1973).

Nevertheless, principal cells of this region are 25-50 μm in height and their nuclei retain their ovoid size and shape (8-10 μm length) and basal location within the cell cytoplasm, but are rotated such that the longitudinal axis of the nuclei is perpendicular to the basement membrane (Kennedy and Heideger, 1979).

1.2.1d. Distal Region:

Principal cells of the distal region of the vas deferens attain a height of 50-90 μm and have long microvilli approximately 25 μm in length. Within the distal region, the overall development of interdigitation between the plasma membranes toward the base of adjacent principal cells range from moderate to extensive and appear to be enhanced when compared with proximal and distal regions. The apical cytoplasm contain large smooth and coated vesicles as well as MVBs, but these components are present in smaller numbers than in principal cells of the proximal region. On the other hand, a relatively larger part of the cytoplasm of the distal segment is occupied by abundant mitochondria, which are smaller, more numerous and have more sinuous elongate profiles than those of the proximal region (Flickinger, 1973). The Golgi apparatus is large and retains its supranuclear location. The

decreased width of the distal region principal cell is accompanied by a change in the orientation of the Golgi complex from horizontal to vertical (Kennedy and Heideger, 1979).The nuclei of principal cells of the most distal segment of the vas deferens are displaced from the basal cytoplasm to an intermediate position within the cell cytoplasm (Kennedy and Heideger, 1979).

However, the most striking feature of the principal cells of the distal region is the presence of large amounts of sER (Flickinger, 1973). Cisternae of the rER are relegated to a relatively smaller area in the basal portion of the cytoplasm than in the cells of the proximal region (Flickinger, 1973). The extensive sER in the principal cells is in two main forms. Tubules of smooth membranes are present as in the proximal region, but they exceed in number by numerous parallel unfenestrated smooth cisternae, which are frequently curved and arranged in a concentric pattern forming whorls of smooth membranes of varying size. Several small whorls less than a micron in diameter are often found in the apical cytoplasm. Large whorls several microns in diameter are present in the perinuclear cytoplasm and sometimes extend into the basal regions normally occupied by the rER. The exact configuration of these smooth membranes is difficult to ascertain, but the precise definition of numerous parallel membranes in many sections suggest that they are cisternae or flat sacs rather than tubules (Flickinger, 1973). The degree of development of these features, especially the whorls of sER, varies from one cell to another in the distal region (Flickinger, 1973). The importance of abundant mitochondria associated with abundant sER have suggested steroid synthesis and will be discussed later.

1.2.1e. Terminal Region:

This is the region of the vas deferens found within the prostate gland. Only Cooper and Hamilton (1977) differentiate this region from the distal region by describing a region of phagocytic cells where the typical tall principal cells and basal cells are replaced by a nest of small randomly-oriented cells that phagocytose spermatozoa.

1.2.2. Basal cells:

Basal cells also appear along the entire vas deferens. These cells which do not reach the lumen, usually insinuate themselves between adjacent principal cells at the base of the

epithelium where they show a large area of contact with the basement membrane (Flickinger, 1973; Robaire and Hermo, 1988). Even though the structure of the complex basement membrane has been studied, neither the relationship between basal plasma membrane and basement membrane nor the presence of hemidesmosomes have been studied (Clermont and Hermo, 1988). Basal cell plasma membranes show interdigitations as well as desmosomes with the lateral plasma membranes of adjacent principal cells (Kennedy and Heideger, 1979). The basal cell cytoplasm, surrounding the large nucleus, contains, in addition to the Golgi apparatus, mitochondria, and a few cisternae of rER, a few lysosomes, MVBs, and the occasional lipid droplet. Though it is apparaent that these cells are not very active, the presence of coated and uncoated pits along the cell surface together with MVBs and lysosomes suggests that these cells are capable of endocytosis (Hamilton, 1975). However, the exact function of basal cells remains unknown. In addition to the variations among the principal cells, the morphology of basal cells, in terms of nuclear size and shape, likewise exhibit regional variation at the light microscope level. Basal cell nuclei within the proximal region are ovoid in shape, 2.5-5 μm in length, with longitudinal axis oriented parallel to the basement membrane, whereas in the distal region, basal cell nuclei are round with a diameter of approximately 4 μm (Kennedy and Heidger, 1979).

The proliferative activity in the rat vas deferens has not been studied but it has been studied in the epididymis where there was a decline in the mitotic and labeling indices of principal and basal cells in rats aged 2.5, 4 and 12 months (Clermont and Flannery, 1970). This suggested that the epididymal cell population (principal and basal cells) is expanding in adult animals and was not renewing itself. Therefore, by extension from the epididymal studies, basal cells could not be considered as stem cells for the epithelial cells of the rat vas deferens.

1.2.3. Clear cells:

Clear or foamy cells were observed in the initial portion of the proximal region of the vas deferens albeit at a reduced number than the cauda epididymidis (Kennedy and Heideger, 1979). Extending from the apical plasma membrane of clear cells into the lumen are several unevenly distributed long, slender microvilli (Hamilton et al, 1977). Clear cells are

characterized by an apical region containing numerous pale stained smooth vesicles of different sizes, a large supranuclear region packed with an abundance of large densely stained lysosomes (cytoplasmic bodies) of various shapes and sizes, and a basal region presenting a variable amount of lipid droplets and a lightly stained nucleus with dispersed chromatin (Robaire and Hermo, 1988). The content of the large cytoplasmic bodies decreases in electron density in a gradient from supranuclear to apical cytoplasm (Kennedy and Heideger, 1979). In addition, the apical and supranuclear regions contain mitochondria, rod- and circular-shaped in profile, which are smaller and have more numerous cristae than do mitochondria of adjacent principal cells. Also present are scattered Golgi profiles, aggregates of ribosomes, and smooth-surfaced cisternae (Kennedy and Heideger, 1979).

1.2.4. Narrow cells:

Also called "Mitochondrion-rich" or pencil cells, they are found in the rat vas deferens (Kennedy and Heidger, 1979). These cells are clearly different in appearance from the neighboring principal cells. They are located toward the luminal surface of the epithelium and are characterized histologically by its goblet-shaped cell body (Niemi, 1965; Hamilton, 1975). The lateral plasma membranes of narrow cells have numerous interdigitations with adjacent principal cells (Niemi, 1965). These cells, few in number, present an apically located heterochromatic and lobulated nucleus, numerous mitochondria, a few indented dense bodies, and MVBs. The stacks of Golgi saccules surround the nucleus, while a few cisternae of rER are scattered about in the cytoplasm (Robaire and Hermo, 1988). The mitochondria of the apical and infranuclear regions of these "mitochondrion-rich" cells are smaller in cross sectional diameter than are the mitochondria from other principal cells (Kennedy and Heideger, 1979). In humans, narrow cells have been reported to be a dying epithelial cell (Hoffer, 1976); in the rat, their function is unknown but they do not appear to be a dying cell type. Narrow cells could correspond to a group of cells in the proximal vas deferens that express both (H^+)-ATPase and carbonic anhydrase II and function in acidifying the lumen of the vas deferens which has a role in maintaining sperm in a quiescent state during their passage through the vas deferens (Breton et al., 1996)

1.2.4. Macrophages:

Intercellular macrophages are characterized by extensive cytoplasmic accumulations of dense or residual material, especially in the basal portion of the epithelium and they presumably gain entrance into the epithelium by migration through the basal lamina. In normal animals, these macrophages usually occur singularly and often contain residues which resemble sperm heads and tail pieces in advanced stages of dissolution, in addition to accumulations of homogeneous and membranous material (Flickinger, 1973; Kennedy and Heideger, 1979).

Similar cell types have been reported in a wide variety of species including monkeys and humans (Popovic et al., 1973; Hoffer, 1976; Ramos, 1979). Many of the features observed in the rat vas deferens have also been found in the human vas deferens. That's why the rat serves as a good model to study human vas deferens.

1.3. Functions of the Vas Deferens:

The vas deferens is apparently not a simple tube for sperm conduit (Niemi, 1965), but seems to be more complex functionally than previously supposed (Flickinger, 1973; Hamilton, 1975). It seems likely that these regional differences in fine structure reflect functional differences in different parts of the vas deferens (Flickinger, 1973). Simple mechanical continuity of the vas deferens alone may not be sufficient for normal fertility and its restoration after vasectomy, but the presence of morphological and functional sequence in the cells of the epithelium may also be important (Flickinger, 1973).

The human epididymis, unlike most species including rat, does not have a bulbous cauda for storing 50-60% of the sperm present in the epididymis (Amann, 1981; Amann and Howards, 1980; Johnson and Varner, 1988; Turner, 1995). Therefore, it has also been suggested in humans that a large portion of the proximal vas deferens does in fact serve in sperm storage (Turner, 1995). Thus sperm would need to be protected and maintained while in this region.

In addition, it has been noted that in efferentiovasostomy (efferent duct-vas deferens anastomoses) and caput epididymovasostomy, sperm were able to achieve natural-mating pregnancies (Schoysman and Bedford, 1986; Silber, 1988, 1989). It was thus suggested that one possibility allowing for sperm maturation after such procedures is that the

microenvironment of the vas deferens is sufficient to allow for the maturation of some sperm cells. However, the function of the epithelial cells in terms of secretion, endocytosis and protecting sperm in this region is unknown.

Vasectomy is a common method of contraception in males and results in major changes to the epididymis in terms of epithelial cell size and lumen diameter, number of lysosomes, infiltration of blood cells, higher antisperm antibody levels and formation of cyst-like granulomas (Flickinger et al., 1995). However, little is known about the functions of the epithelial cells of the normal vas deferens. This is of particular importance following the re-anastomosis of the vas deferens, where complications arise in developing fertile sperm, especially since little is known about what factors are normally involved in creating a normal environment for sperm in the vas deferens. As a result a detailed information on the structure and functions of the vas deferens of normal rats is essential in understanding the effects of vasectomy and vasovasostomy.

In the following sections, five functions of the vas deferens will be discussed including: secretion, water transport, steroid synthesis, protection from electrophiles, and endocytosis. Since the middle vas deferens shares structural features with the proximal and distal vas deferens, in the following sections the vas deferens was functionally subdivided into proximal and distal regions.

1.3.1. Secretion:

As mentioned above, the most prominent feature of the supranuclear region of principal cells especially from the proximal region of the vas deferens is a well developed rER and an elaborate Golgi apparatus formed of the many stacks of saccules and associated small vesicles. The Golgi apparatus is associated with so many vesicles that Niemi (1965) found no clear distinction between the Golgi apparatus and the cytoplasmic vesicles. The stacks of Golgi saccules show on their trans face a close association with many smooth vesicles containing a wispy material and these are involved in transport of materials between the saccules of the Golgi, while wells containing a few vesicles can be found on their cis face (Robaire and Hermo, 1988). On the other hand, some of the Golgi-associated small coated vesicles fuse with the apical cell membrane and could serve either to convey enzymes and/or

surface-coat material to the apical plasma membrane, or to replace membrane lost from the cell surface during protein absorption (Friend and Farquhar, 1967).

The first radioautographic evidence that sugars taken up by the epididymis can be synthesized into glycoproteins via the classical merocrine pathway in this tissue came from early light microscope studies of Neutra and Leblond (1966). They found that after administration of labeled galactose to adult rats, radioautographic silver grains first appeared over the Golgi apparatus and subsequently over the luminal surface of epididymal principal cells. Using labeled fucose, Bennett et al. (1974) confirmed that the rat vas deferens could take up sugars and incorporate them into substances that are secreted into the lumen. Although the cellular apparatus for the synthesis and packaging of proteins is evidently present in principal cells of the vas deferens, Hamilton (1975) has reported a lack of classical secretory granules. Burkett et al. (1987) have shown by lectin-horseradish peroxidase histochemistry that the Golgi and apical zones of principal cells of the mouse vas deferens stain and this was interpreted as evidence for synthesis and secretion of glycoproteins.

In vitro studies performed on isolated epithelial strips showed that epithelium from the proximal vas deferens incorporate more labeled amino acid into cytosolic and incubation medium proteins than do the epithelium from the distal vas deferens (Wenstrom and Hamilton, 1984). This difference in the amount of protein synthesis was suggested to be due to the difference in amount of rER in principal cells from the proximal and distal regions (Wenstrom and Hamilton, 1984). Despite these studies, there has been little work done on the classical merocrine secretion of specific proteins by the individual epithelial cell types.

Sulfated glycoprotein-2 (SGP-2), also designated as clusterin or apolipoprotein J, has been shown to be synthesized by epididymal principal cells (Sylvester et al., 1984, 1991; Tung and Fritz, 1985; Mattmueller and Hinton, 1991; Hermo et al., 1991; Cyr and Robaire, 1992; Tenniswood et al., 1998) and to be similar in molecular weight to the Sertoli-derived SGP-2 except for variations in glycosylation (Sylvester et al., 1984). When anti-SGP-2 antibodies were used in the caput, corpus and proximal cauda epididymal regions, a checkerboard immunostaining pattern was observed in the case of principal cells with cells alongside each other being either intensely, moderately or weakly reactive, suggesting that

cells were out of synchrony with respect to the synthesis and secretion of SGP-2 (Hermo et al., 1991). In the epididymal lumen, a major fraction of SGP-2 was free or loosely associated with sperm, whereas a smaller fraction was more tightly bound (Law and Griswold, 1994). It has been suggested that SGP-2 provides the sperm surface with a protective coat guarding against non-specific proteases, glycosidases or other injurious agents or that it solubilizes and transports lipids from principal cells to the sperm surface (Hermo et al., 1991; Law and Griswold, 1994), although many other functions have been attributed to SGP-2 (Tenniswood et al., 1998). Found in many other tissues, SGP-2 is associated with numerous physiological and pathological conditions (Jenne and Tschopp, 1992). However, the synthesis of SGP-2 in the vas deferens has not been studied.

In addition, apocrine secretion has been demonstrated in the vas deferens of different species involving blebs or protrusions of the apical cytoplasm of principal cells that ultimately detach to be liberated into the lumen (Niemi, 1965; Agrawal and Vanha-Perttula, 1988a; Renneberg et al., 1995). In the mouse, a protein without a signal peptide sequence and glycosylation site limiting it to the cytosol, called mouse vas deferens protein (MVDP), has been postulated to be secreted via this mechanism (Manin et al., 1995). However, little is known about which proteins may be secreted by this method or other possible functions for apocrine secretion.

1.3.2. Water Transport:

Niemi (1965) mentioned in passing that the basal cytoplasm of principal cells is mostly vacuolar and the vacuoles often form vertical clefts. However, he failed to identify these as intercellular dilations and to suggest any functions for them. On the other hand, the venous plexus of the distal vas deferens has been suggested to act as a large pool for diffusion of materials out of the duct (Hamilton and Cooper, 1978). Taken together, these suggest that the vas deferens could be involved in water transport.

Anywhere in the body where there is fluid secretion or absorption, fluid passes across an epithelial barrier quickly and at low energy costs. In all these places the principal driving force appears to be osmotic gradient, generated by active ionic transport, but water flow seems to occur quickly because of the presence of water channels in the apical or the

basolateral membranes of the epithelial cells (Wintour, 1997). These water channels are encoded by homologous genes known as aquaporins (Wintour, 1997).

Aquaporin 1 (AQP-1), also known as channel-forming integral membrane proteins of 28KDa (CHIP28), is the most ubiquitous of all aquaporins and it is found in many tissues and organs (Agre et al., 1993; Wintour, 1997). AQP-1 exists in the membrane as a homotetramer, with each subunit crossing the plasma membrane six times (Agre et al., 1993). AQP-1 is the major water channel of erythrocytes, and epithelial cells of the proximal convoluted tubule and the thin descending limb of the loop of Henle in the kidney (Agre et al., 1993). In the rat male reproductive tract, AQP-1 is located on the brush border and basolateral membranes of nonciliated cells of the efferent ducts (Brown et al., 1993). AQP-1 is also found in plasma membranes of epithelial cells of distal region of the vas deferens, seminal vesicles and prostate but not the cells in seminiferous tubules, epididymis and proximal region of the vas deferens (Brown et al., 1993). This suggests that AQP-1 may be a principal mediator of the transmembrane water transport in the distal vas deferens. However, despite various studies on aquaporins, little has been done on the specific AQP-1 localization in the vas deferens, especially in the vascular channels.

1.3.3. Steroid Synthesis:

As mentioned above, the most prominent feature of the infranuclear region of principal cells especially from the distal region of the vas deferens is abundance of sER forming extensive membrane whorls (Niemi, 1965; Flickinger, 1973; Hamilton, 1975; Kennedy and Heidger, 1979). A variety of functions has been ascribed to sER and the most prominent is steroid synthesis by virtue of the presence of many enzymes of steroid biosynthesis in sER membranes. Another possible function for the extensive sER is that it could provide a storage site for cholesterol either synthesized in situ or adsorbed from the circulation (Fawcett, 1963).

Evidence for cholesterol synthesis comes from in vitro biochemical studies which show significant amount of labeled acetate is incorporated into both cholesterol and testosterone along the entire length of the rat epididymis and vas deferens and that testosterone production is one and half times greater in the vas deferens than in the caput

epididymidis (Hamilton and Fawcett, 1970; Hamilton, 1971). This feature of the vas deferens was attributed to the extensive whorls of sER which was reconstituted by administration of testosterone after castration (Hamilton, et al. 1969) Since the extensive sER is found in the distal region of the vas deferens, Chinoy and Chinoy (1983) found that the distal region was comparatively more affected by castration than the proximal region.

Two of the sER resident enzymes involved in steroid biosynthesis from cholesterol, are Δ^4-3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD). 3β-HSD is a marker for steroidogenic cells such as the interstitial cells of Leydig in the testis (Ewing and Zirkin, 1983). It converts pregnenolone, 17α-hydroxypregnenolone and dehydroepiandrosterone and androstenediol to progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone, respectively (Baillie et al., 1966). While 17β-HSD catalyzes the interconversions of dehydroepiandrosterone to androstenediol and androstenedione to testosterone (Baillie et al., 1966). Demonstration of steroid synthesis in the vas deferens has also been performed by histochemical localization of 3β-HSD and 17β-HSD activities in several species other than the rat (Tingari, 1973; Prakash and Moore, 1982). However, little is known about the localization of 3β-HSD and 17β-HSD in the different regions of the rat vas deferens.

However, for steroids to become soluble in the seminal fluid, they need to be conjugated to sulfur, glucuronic acid, or N-acetylglucosamine and for the epididymis and vas deferens to secrete steroids into the lumen they need to possess these enzymes (Oertel & Treiber, 1968; Hamilton and Fawcett, 1970). Hamilton (1971) measured the conjugation of steroids by rat epididymis and vas deferens and found it to occur in these organs with a greater activity in the vas deferens.

In addition to having the capacity to conjugate steroids, the rat vas deferens has also the capacity to metabolize labeled testosterone into four major steroids: dihydrotestosterone, 5α-androstane-3α -17β diol, Δ^4-androstenedione, and androsterone. There is no significant difference between the proximal and distal regions but immature vas deferens is significantly more active compared to the mature (Kumari et al., 1980; Seethalakshmi et al. 1982).

Other evidence for steroid synthesis by vas deferens comes from measurements of

C19 steroids in the deferential vein which drains the cauda epididymidis and runs along the length of the vas deferens to enter the hypogastric vein (Lewis and Moffat, 1975). The levels of C19 steroids are considerably higher in the deferential vein than in the peripheral blood (radial vein) and are comparable with those in the testicular vein of three dogs (Pierrepoint et al., 1975). Furthermore, the epididymis, in the absence of the ipsilateral testis, provides a greater sustaining influence on the prostate and seminal vesicles than do the testis and epididymis separated from the target organs by vasoligation in the rat (Pierrepoint et al., 1974).

Evidence for epididymis and vas deferens synthesizing androgenic steroids suggests that steroids present in epididymal seminal fluid are derived, at least in part, from the epididymis itself (Hamilton and Fawcett, 1970). However, the functional significance of steroid production in the epididymis and vas deferens is imperfectly understood at present. Steroids, being lipid soluble, could easily affect changes in permeability or perhaps even structure of the sperm plasma membrane (Mounib, 1964) and could also depresses oxygen uptake by spermatozoa (Gassner and Hapwood, 1955; Mounib, 1964). These and other effects of steroids may play some role in sperm capacitation (Hamilton et al., 1969). Since the semen and sperm contain significant amounts of enzymes for breaking down steroid conjugates (Mann, 1964), it seems probable that steroids are metabolized by the sperm as they move down the excurrent duct. The contribution by the epididymis and vas deferens then would act to renew the steroid pool that would be depleted by sperm metabolism (Hamilton and Fawcett, 1970; Hamilton, 1971).

1.3.4. Protection from electrophiles:

As mentioned above, the cauda epididymidis and proximal region of the vas deferens are major storage sites for sperm in many species, including humans (Turner, 1995). The epithelial cells lining these regions must, therefore, play important roles in providing sperm with a suitable environment for their survival and protection. The relatively low temperature of the cauda has been said to facilitate sperm storage by enhancing oxygen availability to the cells (Djakiew and Cardullo, 1986). However, oxygen availability implies increased presence of oxygen radicals and potential for deleterious peroxidation of sperm membranes, especially

considering that they are high in polyunsaturated fatty acids (Nonogaki, 1992). Oxygen radicals are known to damage sperm in the ejaculate (Aitken, 1989) and their presence would presumably mitigate against successful long-term sperm storage in an enhanced oxygen environment. Some researchers have speculated that the cauda lumen fluid contains anti-oxidant molecules such as glutathione that prevents oxygen radical injury (Agrawal and Vanha-Pertulla, 1988b; Hinton and Palladino, 1995).

Glutathione S-transferases (GSTs) are a family of soluble isozymes involved in cellular detoxification. They prevent the build up of potentially toxic substances by catalyzing the conjugation of reduced glutathione with various electrophilic substances. In addition to their main function in detoxification, they also function in steroid isomerization (Benson et al., 1977), glutathione peroxidation (Prohaska and Ganther, 1976), leukotriene C biosynthesis (Pemble et al., 1986), and can bind noncovalently to a number of nonsubstrate ligands including steroids (Ketley et al., 1975; Homma et al., 1986).

The soluble GSTs are dimeric proteins from a multigene family with five classes (alpha, mu, pi, theta, and sigma) grouped according to the amino acid homology of their subunits (Mannervik and Danielson, 1988; Buetler and Eaton, 1992; Daniel, 1993). The alpha gene family contains the Ya, Yc, and Yk subunits, the mu gene family consists of Yb1-4, Yn, and Yo subunits, while the pi gene family consists of Yf subunit. GSTs form homo- or heterodimers from subunits that are members of one gene family but not between gene families (Mantle et al., 1990). GSTs have similar molecular weights but differ with respect to isoelectric point and substrate specificities (Jakoby et al., 1976). Cells have evolved such a vast number of GSTs to protect themselves against a wide variety of potentially harmful substances that they can encounter.

Hales et al. (1980) have shown that the epididymis-vas deferens contains high GST activity, approximately 50% of that found in the liver on a per-mg of protein basis, and that there are a large number of transferases in the epididymis-vas deferens which are differentially localized along the length of this tissue. Furthermore, the activities of the GSTs in the epididymis have been shown to be androgen dependent and often region specific (Robaire and Hales, 1982). These enzymes have been suggested to protect the epididymis-

vas deferens and maturing sperm from harmful electrophiles (Hales et al., 1980).

Recently, the distribution of different GST subunits has been examined with light and electron microscopic immunocytochemistry along the epididymis of the adult rat. Yf expression is distributed in a region specific manner in principal and basal cells, with principal cells showing a decrease in staining down the duct while basal cells become intensely reactive in the corpus and proximal cauda regions (Veri et al., 1993). In contrast, Yo expression in principal cells increases in intensity from the initial segment to the cauda epididymidis, while basal cells were unreactive throughout the epididymis (Veri et al., 1994). Like the Yf and Yo subunits, the Yc, Yb1, Ya, and Yb2 show region specific expression in principal and basal cells with each often differing from the other (Papp et al., 1995). The absence of reactivity in one cell type of a given region is usually compensated for by the reactivity in the other cell type. The varied GST expression in these cells along the epididymis is thought to ensure that sperm would be protected from a wide variety of blood-borne electrophiles as they traverse the duct and during storage in the cauda epididymidis (Papp et al., 1995).

While the expression of different GST has been examined in the epididymis up to the proximal cauda region (Papp et al., 1995), little is known about their cell and region specific distribution in the middle and distal cauda epididymidis as well as the different regions of the vas deferens.

1.3.5. Endocytosis:

As mentioned above, the most prominent feature of the apical cytoplasm of principal cells, especially from the proximal region of the vas deferens is large numbers of coated and uncoated pits, small coated and uncoated vesicles of similar size (60 nm), large coated and uncoated vesicles (100 nm), MVBs and lysosomes (Niemi, 1965; Friend and Farquhar, 1967; Flickinger, 1973; Hermo and De Melo, 1987).These structural features together with the long microvilli that increase surface area have been suggested to function in absorption of material from the lumen (Neimi, 1965; Flickinger, 1973).

The first evidence for endocytosis comes from a study by Friend and Farquhar (1967) who found that the introduction of protein material (horseradish peroxidase) into the lumen

of the vas deferens and its subsequent uptake appear to trigger the synthesis and movement of lysosomal enzymes from Golgi apparatus to lysosomes, since increased numbers of small coated vesicles are seen near MVBs at 40 minutes after peroxidase injection. By definition, the small coated vesicles correspond to primary lysosomes since they carry lysosomal enzymes.

From their study, Friend and Farquhar (1967) proposed a mechanism where horseradish peroxidase is taken up in bristle-coated invaginations of the apical cell membrane. These invaginations pinch off and became large (100 nm) coated vesicles in the apical cytoplasm. The large coated vesicles lose their coat, become smooth, and subsequently fuse with, and discharge their peroxidase content into the MVBs. The preoxidase content of the latter increases as a function of time. Concomitantly, there is a doubling in the number of small coated vesicles and a shift in their distribution from the Golgi region to the apical cytoplasm near MVBs and the apical cell membrane with which they apparently fuse.

Hermo and De Melo (1987) provided further evidence for endocytosis in the principal cells of the vas deferens. In addition, they found evidence for transcytosis of substances from the lumen to the lateral intercellular space.

Therefore, the lumen of the cauda epididymidis and vas deferens is not only modified by the secretion of proteins therein, but also by transcytosis and/or endocytosis of substances via coated pits on the apical surface of principal cells and the subsequent appearance of tracers in the endocytic apparatus composed of endosomes, MVBs and lysosomes where they are presumably degraded (Friend and Farquhar, 1967; Friend, 1969; Moore and Bedford, 1979; Hermo and De Melo, 1987). While lysosomes are abundant in principal cells (Hamilton, 1975), and studies have documented a cell and region specific distribution of lysosomal integral membrane proteins (Suarez-Quian et al, 1992), the nature of the soluble lysosomal proteins has not been analyzed, nor have receptors on the apical cell surface of principal cells for receptor-mediated endocytosis been described.

Soluble lysosomal proteins include a group of proteolytic enzymes named cathepsins which play an important role in the intracellular degradation of exogenous and endogenous proteins (Kirschke et al., 1980; Kominami et al., 1991) and activation of enzyme precursors

(Barrett and Kirschke, 1981). There is also evidence that cathepsins play a limited role in antigen processing and inflammation, contributing to the overall immune response (Takahashi et al., 1989). In addition to their function in normal cells, cathepsins are also involved in metastasis of cancer cells (Domagala et al., 1992; Rochefort, 1994).

Of the many cathepsins thus far identified, only cathepsins A, D and B will be disctussed in this thesis. Cathepsin A, also known as protective protein, is a lysosomal glycoprotein forming a high molecular weight complex with lysosomal β-galactosidase and neuraminidase (Satake et al., 1994). In this complex cathepsin A functions as a protective protein to regulate the expression of these enzymes in lysosomes by stabilizing β-galactosidase and activating neuraminidase (Galjart et al., 1991; Itoh et al., 1995), in addition to catalytic activities for the hydrolysis of peptide and ester bonds such as those of acid carboxypeptidase, neutral esterase and carboxyl-terminal deamidase (Galjart et al., 1991; Itoh et al., 1995). Cathepsin D, an aspartyl endopeptidase with a molecular weight of 42KDa and optimal activity at pH 3.8, has been studied in many tissues where it has been localized in lysosomes (Srivastava and Ninjoor, 1982; Kominami et al., 1991). Cathepsin B, a cysteine proteinase with optimal activity at pH 5.5-6.5, is present in lysosomes of many cell types (Scott et al., 1987; Yokota and Kato, 1988; Kominami et al., 1991). In the epididymis, region specific expression for principal, clear and basal cells have been noted for synthesis of cathepsins A, D and B (Igdoura et al., 1995; Hermo, unpublished results). However, these lysosomal proteins have not been localized in the different regions of the cauda epididymis and vas deferens.

Sulfated glycoprotein-1 (SGP-1) is another lysosomal protein that is heavily glycosylated and sulfated (Sylvester et al., 1989). It has four domains that share substantial sequence similarity with the precursor of human sphingolipid activator proteins called prosaposin (Morimoto et al., 1988). Present in lysosomes, prosaposin is proteolytically cleaved into four smaller proteins called saposins A, B, C, and D, each of which are believed to solubilize certain lipids so that they may be acted upon by specific lysosomal hydrolases (O'Brien and Kishimoto, 1991). Absence of saposins leads to various clinical conditions such as Gaucher disease and metachromatic leukodystrophy (Stevens et al., 1981;

Christomanou et al., 1986). SGP-1 shows cell and region-specific expression in the epididymis, but it has not been localized in the different regions of cauda epididymis and vas deferens (Hermo et al., 1992a).

Low density lipoprotein receptor related protein-2 (LRP-2), also known as gp330 or megalin, is a member of a family of endocytic receptors related to the low density lipoprotein receptor, all of which mediate cellular internalization and lysosomal degradation of various ligands (Kounnas et al., 1993, 1994). LRP-2 is highly expressed in coated pits, vesicles and endosomes of various epithelial cells and has been shown to bind SGP-2 with high affinity (Kerjaschki and Farquhar, 1982; Kounnas et al., 1995; Zheng et al., 1994). In the epididymis, LRP-2 was localized to the apical surface of principal cells where it was suggested to mediate the uptake of SGP-2 (Morales et al., 1996). Together with the finding that principal cells of the intermediate zone not only secreted but endocytosed SGP-2, it was suggested that SGP-2 may be actively turned over in the epididymal lumen after performing its function(s) with sperm (Hermo, 1995). However, LRP-2 has not been localized in the different regions of cauda epididymidis and vas deferens.

1.4. Objectives:

The purpose of this thesis is to systematically examine the structure and functions of epithelial cells along the length of the rat vas deferens. Structural features will be examined using routine glutaraldehyde-fixed Epon-embedded material for electron microscopy. Four hypotheses about the functions of the rat vas deferens will be tested using Bouin-fixed paraffin-embedded material for light microscope immunocytochemistry. The first hypothesis is that the vas deferens is associated with water transport (ultimately leading to concentration of sperm). If this is true, it is predicted that pumps or channels shown to be associated with waster transport in other systems may be concentrated or present in either the epithelium or associated tissues of the vas. To test this, AQP-1 will be immunolocalized. The second hypothesis is that the vas deferens is involved in steroid synthesis to provide steroids to maturing spermatozoa in the lumen or to secrete to the circulation. If this is true, it is predicted that enzymes involved in steroid synthesis in other systems should also be found in the epithelial cells of the vas deferens. To test this, 3β-HSD, one such enzyme involved

in steroid synthesis, will be immunolocalized in the vas deferens. The third hypothesis is that the vas deferens is involved in sperm protection from harmful electrophiles while the sperm is stored and/or transported through it. If this is true, it is predicted that enzymes involved in protection from electrophiles such as GSTs in the epididymis should also be found in the vas deferens. To test this, Ya, Yc, Yb1, Yf, and Yo subunits of GSTs will be immunolocalized in principal and basal cells in each of the major subdivisions of cauda epididymidis and vas deferens. The fourth hypothesis is that the vas deferens is involved in secretion and endocytosis of proteins. If this is true, it is predicted that proteins secreted by epithelial cells in the epididymis, should also be secreted from the vas deferens. To test this prediction, the expression of SGP-2, a secretory protein, will be studied. It is also predicted that endocytic receptors and lysosomal enzymes found in the epididymis should also be found in the vas deferens. To test the hypothesis of endocytosis, the expression of cathepsins A, D, and B, and SGP-1, which are lysosomal enzymes and the expression of LRP-2 which is an endocytic receptor will be studied. The first two functions together with the structural features will be examined in the second chapter, while the third function in the third chapter and the fourth in the fourth chapter.

CHAPTER TWO:

Principal Cells of the Vas Deferens are Involved in Water Transport and Steroid Synthesis in the Adult Rat

(Andonian S, Hermo L. *J Androl* 1999a)

2.1. ABSTRACT:

Principal cells show marked structural differences in the proximal, middle, and distal regions of the vas deferens reflective of diverse functional activities. In the present study, their structural features were examined in the electron microscope of glutaraldehyde-fixed, Epon-embedded material, while functional parameters were examined by light microscope immunocytochemistry of Bouin-fixed, paraffin-embedded material. In the proximal region, the cuboidal principal cells resembled those of the cauda epididymidis but few clear cells and occasional narrow cells were present. In the middle region, principal cells often contained blebs of their apical cytoplasm containing vesicular and tubular profiles. These blebs extended far from the cell surface and appeared to be liberated into the lumen suggesting an apocrine type of secretion. In the distal region, dilated intercellular spaces containing numerous membranous profiles of different shapes and sizes were noted between adjacent principal cells and overlying basal cells. The use of an anti-aquaporin-1 antibody revealed an intense reaction over the endothelial cells of numerous vascular channels in the lamina propria. Taken together, these observations suggested water transport from the lumen of the vas deferens via the dilated spaces to underlying vascular channels, the function of which may be to concentrate sperm. The infranuclear cytoplasm of principal cells of this region showed whorls of smooth endoplasmic reticulum (sER). Large intracytoplasmic cavities were found within the sER aggregates and contained membranous profiles which appeared to peel off from the surrounding sER elements. Various images of such cavities closely juxtaposed to the lateral plasma membrane suggested that the membranous profiles of the intercellular spaces were derived from them. Use of anti-3β-hydroxysteroid dehydrogenase antibody revealed an intense reaction over principal cells of the vas deferens, as well as over the blebs in the vas deferens lumen, indicative of steroid synthesis by these cells. The release of sER membranous profiles into the dilated spaces and presence of blebs in the lumen may represent a means of transporting steroids out of principal cells destined for different sites. Steroids in the blebs would be destined ultimately for utilization by luminal sperm, while that of the dilated spaces for muscle layers of the lamina propria. In summary, principal cells of the vas deferens appear to be involved in synthesis and secretion of steroids and eliminating

water from the vas deferens lumen.

2.2. INTRODUCTION:

The vas deferens in the rat, approximately 6 cm long, originates as a straight tube from the coiled cauda epididymidis in the scrotum and ends as the ejaculatory duct within the prostate gland (Hamilton and Cooper, 1978). It has been divided into proximal and distal regions in addition to a terminal portion (Hamilton and Cooper, 1978). The epithelium lining the entire vas deferens is made up of principal cells which show an increase in cell height, microvilli and infranuclear cytoplasm from proximal to distal regions (Niemi, 1965). Basal cells also appear along the entire vas deferens, while clear cells and mitochondria-rich cells have been observed in specific regions (Flickinger, 1973; Kennedy and Heidger, 1979). Similar cell types have been reported in a wide variety of species including monkeys and humans (Popovic et al., 1973; Hoffer, 1976; Ramos, 1979).

Principal cells have a well developed Golgi apparatus and rough endoplasmic reticulum and are recognized as active secretory cells involved in synthesis and secretion of glycoproteins via the classical merocrine type of secretion (Wenstrom and Hamilton, 1984; Burkett et al., 1987; Robaire and Hermo, 1988). In addition, apocrine secretion has been demonstrated in the vas deferens of different species involving blebs or protrusions of the apical cytoplasm of principal cells that ultimately detach to be liberated into the lumen (Niemi, 1965; Agrawal and Vanha-Perttula, 1988; Renneberg et al., 1995).

Over the years it has been shown that principal cells of the distal region of the vas deferens have an abundance of smooth endoplasmic reticulum (sER) in their cytoplasm (Hamilton, et al. 1969; Flickinger, 1973; Kennedy and Heidger, 1979; Robaire and Hermo, 1988) suggesting a role in steroid synthesis (Hamilton and Fawcett, 1970; Hamilton, 1971; Seethalakshmi et al. 1982).

Principal cells of the vas deferens are also active endocytic cells involved in the internalization of substances from the lumen with their subsequent degradation in lysosomes or transcytosis to the lateral intercellular space (Friend and Farquhar, 1967; Hermo and De Melo, 1987). In addition, aquaporin-1 (AQP-1)(CHIP28), a specific water channel protein forming aqueous pores across the plasma membrane has also been identified in the epithelial cells of the ampulla of the vas deferens suggesting transmembrane water transport in this

tissue (Brown et al., 1993).

The purpose of the present study was to examine systematically the structure and functions of principal cells along the length of the rat vas deferens using routine glutaraldehyde-fixed Epon-embedded material for electron microscopy, as well as Bouin-fixed paraffin-embedded material for light microscope immunocytochemistry. The results show region-specific differences in the structural features of principal cells along the duct including apical blebs, abundant sER and related intracytoplasmic cavities, as well as dilated intercellular spaces containing membranous profiles between adjacent principal cells. Immunocytochemical localization of 3β-hydroxysteroid dehydrogenase (3β-HSD) and aquaporin-1 (AQP-1) indicate a role for these cells in steroid metabolism and water transport across the epithelium.

2.3. MATERIALS AND METHODS:

2.3.1. Animals:

Six adult male Sprague Dawley rats (350-450 g) obtained from Charles River Laboratories (St. Constant, PQ) were anesthetized with sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON). The vas deferens of each side was fixed by perfusion in an anterograde manner through the abdominal aorta with 5% glutaraldehyde buffered in sodium cacodylate (0.1M) containing 0.05% $CaCl_2$ at pH 7.4. After perfusing for 10 minutes, the tissues were removed. Grossly, the vas deferens was taken to be the straight tube emerging from the distal coiled loops of the cauda epididymidis. It was arbitrarily divided into three regions, i.e. proximal, middle and distal, of approximately equal length (2 cm) with each being subdivided into proximal and distal halves. The distal region of the vas deferens included a portion that was embedded in the prostate gland. The tissue from each region was cut into small 1 mm^3 pieces and placed in the same fixative for additional 2 hrs at 4 °C. After an overnight wash in buffer, the tissue was postfixed in potassium ferrocyanide-reduced osmium tetroxide (Karnovsky, 1971) for 1 hr to enhance the staining of membranes. The tissue was then rinsed several times in cacodylate buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Sections (0.5–1μm) were cut, stained with toluidine blue and examined under the light microscope. Thin sections of selected areas were cut with a diamond knife, placed on copper grids, and counterstained with uranyl acetate and lead citrate and examined with a Philips 400 electron microscope.

2.3.2. Light Microscope Immunocytochemistry.

2.3.2a. Tissue Preparation:

Four adult male Sprague Dawley rats (350-450 g) obtained from Charles River Laboratories (St. Constant, PQ) were anesthetized with an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON), and their vas deferens fixed by perfusion through the abdominal aorta with Bouin's fixative for 10 minutes. After perfusion, the vas deferens was removed and immersed in Bouin's fixative for another 24 hrs. Prior to immersion, the tissue was divided into proximal, middle, and distal regions so that the entire vas deferens would fit into one paraffin block. After fixation, the tissue was

dehydrated and eventually embedded in paraffin.

2.3.2b. Immunoperoxidase Staining:

Immunoperoxidase staining of vas deferens sections was carried out according to the procedure of Oko and Clermont (1989). Polyclonal anti-aquaporin 1 (AQP-1) (CHIP28) antibody and anti-3β-hydroxysteroid dehydrogenase (3β-HSD) antibody were used at a dilution of 1:100 in Tris-buffered saline (TBS), pH 7.4. The rabbit anti-aquaporin 1 antibody was obtained from Alpha Diagnostics Int. (San Antonio, TX), while the anti-3β-HSD antibody was obtained from Dr. Van Luu-Thé (Laval University, PQ). Details of the preparation and specificity of the anti-3β-HSD antibody can be found in Luu-Thé et al. (1989).

Paraffin sections, 5 µm thick, were deparaffinized in xylene and hydrated in a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished in 70% ethanol containing 1% (v/v) H_2O_2. Once hydrated, the tissue sections were washed in distilled H_2O containing glycine to block free aldehyde groups.

Before immunostaining, the sections were blocked for 15 minutes with 10% goat serum in Tris Buffered Saline (TBS). This and subsequent treatments were accomplished by placing 100 µl of a solution onto a coverslip and overturning the tissue face of the slide onto the drop, thus ensuring that the entire tissue was treated with minimal fluid (Oko and Clermont, 1989). Coverslips were removed by dipping the slides in TBS containing 1% Tween-20 (TWBS). Sections were then incubated in a 37°C humidified incubation chamber for 1.5 hrs with the appropriately diluted primary antibody. After four 5 min washes in TWBS, sections were once again blocked with 10% goat serum in TBS. They were then incubated for 30 min (at 37°C) with goat anti-rabbit IgG conjugated to peroxidase (Sigma; St. Louis, MO) diluted 1:250 in TBS. This was followed by four 5 min washes in TWBS.

The final reaction product was achieved by incubating the sections for 10 min in 500ml of TBS containing 0.03% H_2O_2, 0.1M imidazole, and 0.05% diaminobenzidine tetrahydrochloride (Sigma), pH 7.4. Slides were then washed in distilled H_2O and counterstained with 0.1% methylene blue for 1 min. The tissue was dehydrated by passing

slides through a graded ethanol series, after which the sections were immersed in xylene and mounted with Permount. Specificity of the immunolabeling was confirmed in all tissues by incubation without the primary antibody and use of normal rabbit serum.

2.4. RESULTS:

2.4.1. Structural appearance of the different regions of the vas deferens:

2.4.1.1. Light microscope:

The vas deferens of the rat, measuring approximately 6 cm at its origin from the distal coiled tubules of the cauda epididymidis to its entry into the prostate, was subdivided into equal thirds designated as proximal, middle and distal regions, respectively. In the proximal region, the epithelium was cuboidal in appearance and consisted of principal cells, basal cells, and the occasional clear and narrow cells (Figs.1,2). The epithelium rested on a thin lamina propria composed of several concentric layers of closely apposed flattened myoid cells, subjacent to which were smooth muscle cells (Figs.1,2).

The epithelium of the middle region of the vas deferens was composed of principal and basal cells. Principal cells showed a uniform layer of tall microvilli apically, while dilated intercellular spaces were noted basally between adjacent principal cells and overlying basal cells (Figs.3,4). Their nuclei were located in the mid region of the cell. Infranuclearly, the principal cell cytoplasm appeared as a homogeneous aggregated mass of material. Apically, blebs of cytoplasm of principal cells at times protruded into the layer of microvilli (Figs.3,4). The epithelium rested on a thick lamina propria composed of alternating incomplete concentric layers of fibrocytes and connective tissue components. Numerous capillaries were seen next to the basement membrane and large vascular channels were seen separating the lamina propria from the subjacent myoid cell layers (Figs.3,4). Gradual changes in height of the epithelium and the appearance of the dilated intercellular spaces basally between adjacent principal cells were noted between the proximal and distal halves of the middle vas deferens (Fig.3).

In the distal region of the vas deferens, the height of principal cells increased and the dilated intercellular spaces were especially prominent (Figs.5,6). The latter were of various sizes and shapes, often extending up to the mid point of the epithelium. Nuclei of principal cells were in the mid region of the cell, while homogeneous aggregated masses of material occupied the basal region of the cytoplasm (Figs.5,6). Microvilli formed a thick layer at times interrupted by apical blebs of principal cells. The lamina propria showed numerous

capillaries next to the basement membrane and layers of fibrocytes alternating between connective tissue components. The deeper layer of the lamina propria was filled with large vascular channels, subjacent to which were the myoid cell layers.

2.4.1.2. Electron microscope:

2.4.1.2a. Epithelium:

In the proximal vas deferens, the supranuclear region of principal cells showed coated pits, endosomes, multivesicular bodies and lysosomes (Fig.7). Numerous stacks of Golgi saccules were present, as well as a plethora of small interconnecting tubular elements possibly corresponding to smooth endoplasmic reticulum (sER) (Fig.7). The basal region of principal cells contained numerous flattened strands of rough ER next to the basally located nucleus (Fig.8). The lateral plasma membranes of adjacent principal cells were closely apposed and interdigitated with one another and neighboring basal cells (Fig.8).

In addition to principal cells, narrow and clear cells were noted in the proximal vas deferens, but neither were numerous. Narrow cells were small in size with a nucleus in the mid position of the cell. The apical region was filled with small vesicles, mitochondria and occasional endosomes and lysosomes (Fig.9A). Clear cells were larger in size and contained numerous endosomes, dense lysosomes and a plethora of small apical vesicles (Fig.9B).

In the middle vas deferens, principal cells revealed similar morphological features in their apical/supranuclear region as compared to the proximal vas deferens with the exception of numerous mitochondria in their apical region (Fig.10A). Also more apparent was the presence of blebs of cytoplasm emanating from the apical region of principal cells (Figs.10A,B). Such blebs often extended for some distance into the lumen, with microvilli projecting from their surface. They contained mainly filaments, and small vesicular and tubular elements. Images of blebs apparently detached from the principal cell and loose in the lumen were also noted (Fig.10C). The lumen of the middle vas deferens often contained small flattened and spherical membranous profiles amongst the microvilli of principal cells (Fig.10C). Such blebs were also noted in the distal vas deferens, but less frequently.

The middle vas deferens revealed major structural differences in the basal region of the epithelium as compared to the proximal vas deferens with the abrupt appearance of

dilated intercellular spaces between adjacent principal cells at approximately its mid point. For this reason this region was subdivided into a proximal and distal half. In fact features of principal cells of the proximal half closely resembled those of the proximal vas deferens. However, the distal half of the middle vas deferens was similar in appearance to the distal vas deferens and will thus be discussed together. The major distinguishing features of the distal half of the middle vas deferens and distal vas deferens were: 1) the position of the nucleus at the mid point of the cell; 2) numerous sER elements; 3) dilated intercellular spaces between adjacent principal cells and overlying basal cells which often extended up to the mid point of the epithelium; 4) apical blebs.

The dilated intercellular spaces were of variable shapes and sizes (Figs.11-13). In the absence of close apposition of principal cells, their finger-like processes were readily apparent which closely aligned up against each other or showed focal points of contact ensuring continued contact between principal cells (Figs.11-13). In areas of dilated spaces, close approximation between principal cells were also observed as were close contacts between principal and basal cells (Figs. 11-13). The spaces contained empty looking spherical and irregularly shaped membranous profiles of different sizes (Figs.11-13).

The sER consisted of flattened parallel sheets layered upon each other and at times forming concentric whorls (Figs.11-14). In face view they appeared as an anastomotic tubular network (Fig.13). They filled the infranuclear region of the principal cell along with scattered mitochondria and glycogen granules (Figs.11,12, 14C). In some areas of sER aggregates, large intracytoplasmic cavities were present surrounded by sER elements. These cavities were located deep in the cytoplasm or close to the lateral plasma membrane of the principal cell (Figs.11-14). Some cavities were surrounded by dilated sER elements which appeared to peel off into the interior of the cavity amongst other membranous profiles contained therein (Figs.12,14A,B). Other cavities were spanned by parallel layers of slightly dilated sER elements (Fig.14C). In some cases, cavities were found close to the lateral plasma membrane but separated from it by sER elements and cytoplasmic matrix (Fig.14B), while in other cases the cavity and its membranous profiles were separated from the intercellular space by only the lateral plasma membrane (Fig.14D). The lateral plasma membrane of

principal cells at certain sites adjacent to the dilated spaces showed small projections beneath which sER elements were closely apposed and dilated (Fig.14C). Some images even suggested that the contents of the cavities were being released into the dilated spaces with apparent disruption of the lateral plasma membrane (Fig.13). The contents of the cavities were similar to those seen in the dilated intercellular spaces (Figs.12,13,14A-C).

Basal cells of all regions appeared similar in morphological appearance to each other, however, while they contacted the basement membrane, they also showed focal points of contact with adjacent basal and principal cells in areas of dilated intercellular spaces (Fig.11). Finger-like processes of basal cells in the dilated spaces interdigitated with those of principal cells. Basal cells showed no intracytoplasmic cavities.

2.4.2.2b. Lamina Propria:

Subjacent to the basement membrane of the epithelium of the proximal vas deferens were several alternating myoid cell and connective tissue layers (Fig.15A). Myoid cells showed an elongated nucleus and a cytoplasm filled with filaments, while the connective tissue layers were comprised mainly of collagen fibers (Fig.15A). This was followed by smooth muscle layers. In the middle vas deferens, several discontinuous layers of fibrocytes interspersed between collagen fiber layers were present subjacent to the basement membrane, followed by alternating myoid cell and collagen fiber layers, beneath which the characteristic smooth muscle layers were present (Fig.15B). The pattern was similar in the distal vas deferens, with the exception that the fibrocyte and connective tissue collagen layers were separated from the myoid cell layers by a large vascular plexus which was characteristic of this region (Fig.16). The extent of this plexus was well visualized at the light microscope level (Fig.16 inset).

2.4.2. Light microscope immunocytochemical studies:

The presence of large dilated intercellular spaces between adjacent principal cells suggested the passage of water from the lumen to the underlying lamina propria. Use of anti-AQP-1 antibodies revealed an intense immunoreaction over the endothelium of the subepithelial vascular plexus of the vas deferens (Fig.17A). No reaction was seen over the

endothelium in control slides lacking primary antibody (Fig.17B).Use of anti-3β-HSD antibodies revealed an intense immunoreaction over the epithelium of the vas deferens in relation to principal cells (Figs.18A-C). In addition, staining of principal cells with this antibody enhanced the appearance of the apical cytoplasmic blebs which revealed their irregular shapes, detachment from the cell surface and apparent presence in large amounts free in the lumen of the duct (Figs.18A-C).

2.5. FIGURES AND LEGENDS

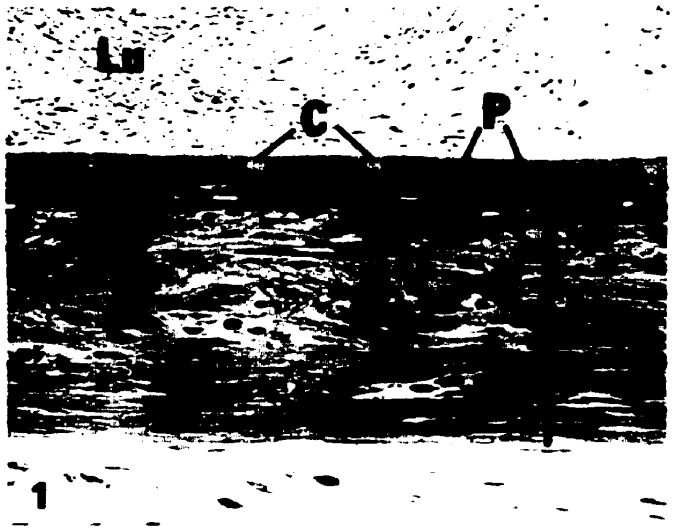
Fig. 1: Light micrograph of a section through the proximal region of the vas deferens showing cuboidal epithelium consisting mainly of principal (P) and occasional clear (C) and narrow (large arrowheads) cells. The epithelium rests on a thin lamina propria consisting of several layers of myoid cells (arrows), subjacent to which are multiple layers of smooth muscle cells (SM). Lu, lumen. X 300.

Fig. 2: Light micrograph of a section through the proximal region of the vas deferens. The cuboidal epithelium consists of principal (P), basal (small arrowheads) and a few narrow (large arrowheads) cells. The underlying lamina propria (LP) contains several alternating concentric connective tissue and myoid cell layers, beneath which are smooth muscle cell layers (SM). Lu, lumen. X 375.

Fig. 3: Light micrograph of the proximal half of the middle region of the vas deferens. The epithelium consists of columnar principal (P) and basal (small arrowheads) cells. Principal cells present tall microvilli (solid star), and in the right part of the field, large dilated intercellular spaces (large arrows) exist between these cells. Note blebs emanating from the surface of some principal cells (open arrows). The epithelium rests on a lamina propria composed of fibrocytes (small arrows), capillaries (large arrowheads) and vascular channels (va). Lu, lumen. X 375.

Fig. 4: Light micrograph of the distal half of the middle vas deferens. Principal cells (P) are taller than the proximal half of the middle vas deferens. Their nuclei (n) are located in the mid portion of the cell. Microvilli (solid star) form a thick homogeneous layer at the apex of the epithelium. In the basal region of the epithelium, dilated intercellular spaces (large arrows) exist between adjacent principal cells and encompassing basal cells. The infranuclear cytoplasm of principal cells appears as a uniform aggregated mass of material (open stars). The lamina propria shows capillaries (large arrowheads) immediately subjacent to the epithelium and layers of fibrocytes (small arrows) and vascular channels (va). X 375.

Fig. 5: Light micrograph of the distal half of the distal vas deferens. Principal cells (P) are taller than in the preceding region, and the dilated intercellular spaces (large arrows) are more prominent, of different shapes and sizes and at times extend up to the mid point of the



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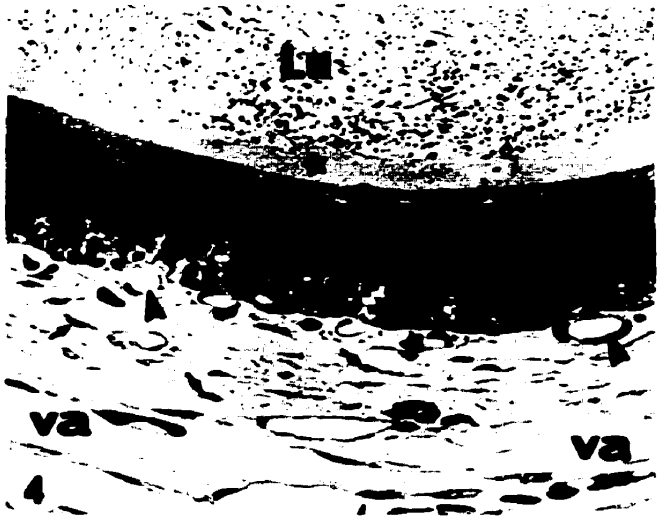

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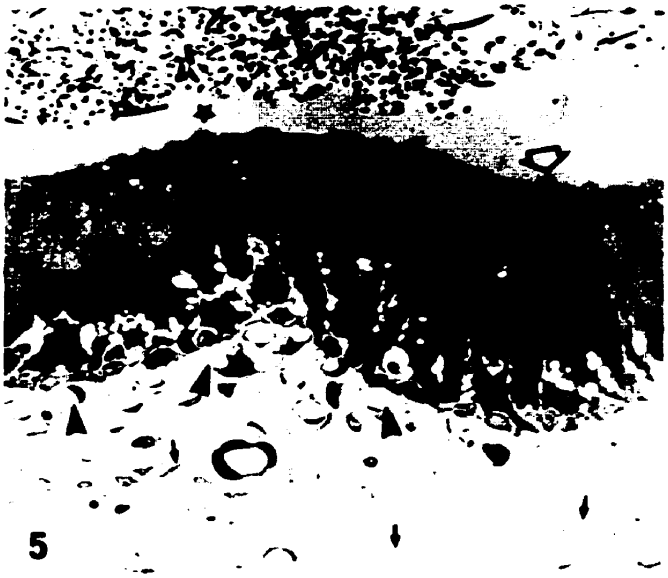

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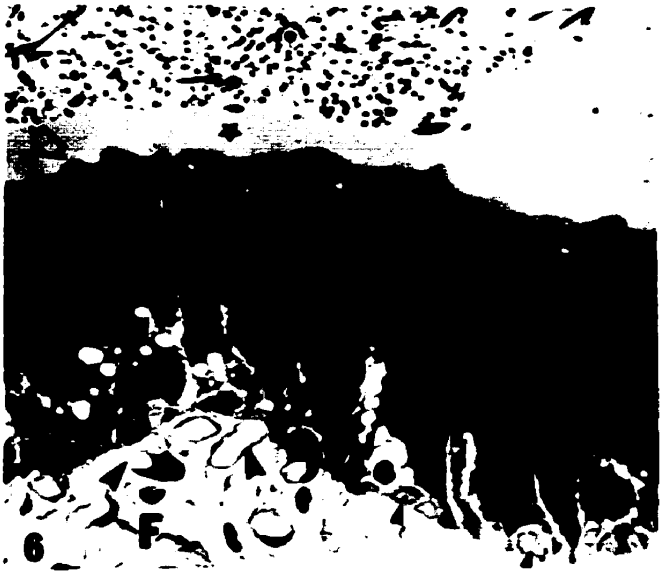

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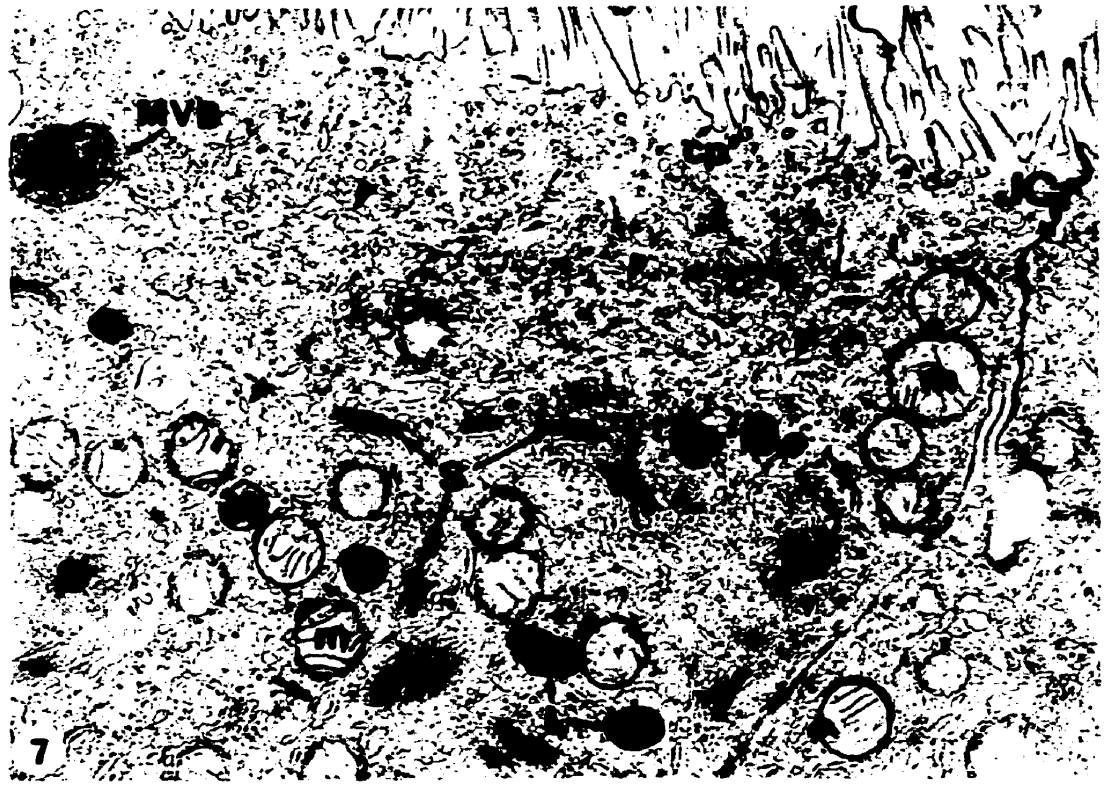
Fig. 7: Electron micrograph of the apical and supranuclear cytoplasm of a principal cell of the proximal region of the vas deferens. Several stacks of saccules (S) of the Golgi apparatus, mitochondria (m), multivesicular bodies (MVB) and lysosmes (L) are evident. A plethora of cisternae of smooth endoplasmic reticulum which appear as small irregular tubular profiles form an extensive anastomotic network in this region of the cytoplasm (arrowheads). Note junctional complex (JC) between adjacent principal cells and coated pits (cp) at the apical cell surface. Arrows, rough endoplasmic reticulum. X 15,400.

Fig. 8: Electron micrograph of the basal cytoplasm of adjacent principal cells of the proximal region of the vas deferens reveals long parallel cisternae of rough endoplasmic reticulum (arrows) and occasional mitochondria (m). Note basal position of nuclei (N) of principal cells in this region. The lateral plasma membranes of adjacent principal cells are closely apposed to each other and show numerous interdigitations (arrowheads). Adjacent basal cells (Ba) are noted, one of which shows an elaborate Golgi apparatus (G). The lamina propria consists of myoid cell layers (My) interposed between layers of collagen fibers (Co). X 9,890.

epithelium. Their infranuclear cytoplasm contains an aggregated mass of material (open stars). Microvilli (solid star) form a thick layer at times interrupted by occasional blebs of principal cells (open arrow). Numerous capillaries (large arrowheads) abound close to the base of the epithelium, while the lamina propria contains fibrocytes (small arrows).X 300.

Fig. 6: Light micrograph of the distal half of the distal vas deferens. In the base of the epithelium, large dilated intercellular spaces of various shapes and sizes (large arrows) exist between adjacent principal cells (P) and overlying basal cells (small arrowheads) and at times extend up to the mid point of the epithelium. Note numerous capillaries (large arrowheads) immediately subjacent to the epithelium. Cytoplasmic blebs (open arrow) of principal cells appear in the thick homogeneous layer of microvilli (solid star). The infranuclear cytoplasm of principal cells consists of homogeneous aggregated masses of material (open stars). F, fibrocytes; n, nuclei of principal cells. X 375.


MVB
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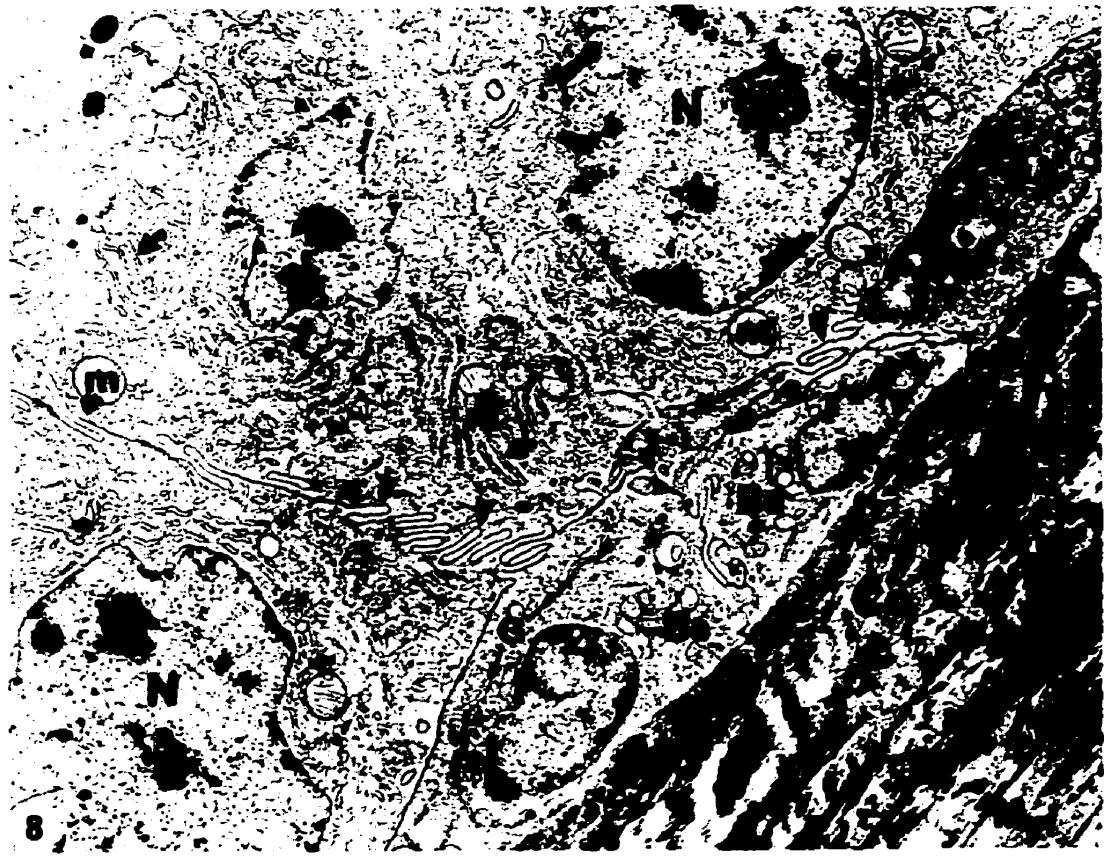
N
N
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Fig. 9: Narrow and clear cells of the proximal region of the vas deferens. **(A)** The narrow cell, although filled with small apical vesicles (arrowheads), contains few lysosomes (L). The nucleus (N) is positioned high in the epithelium. **(B)** The clear cell is filled with numerous small apical endocytic vesicles (arrowheads), endosomes (E) and supranuclear lysosomes (L). Lu, lumen; N, nucleus; P, principal cells. A: X 12,650; B: X 15,050.

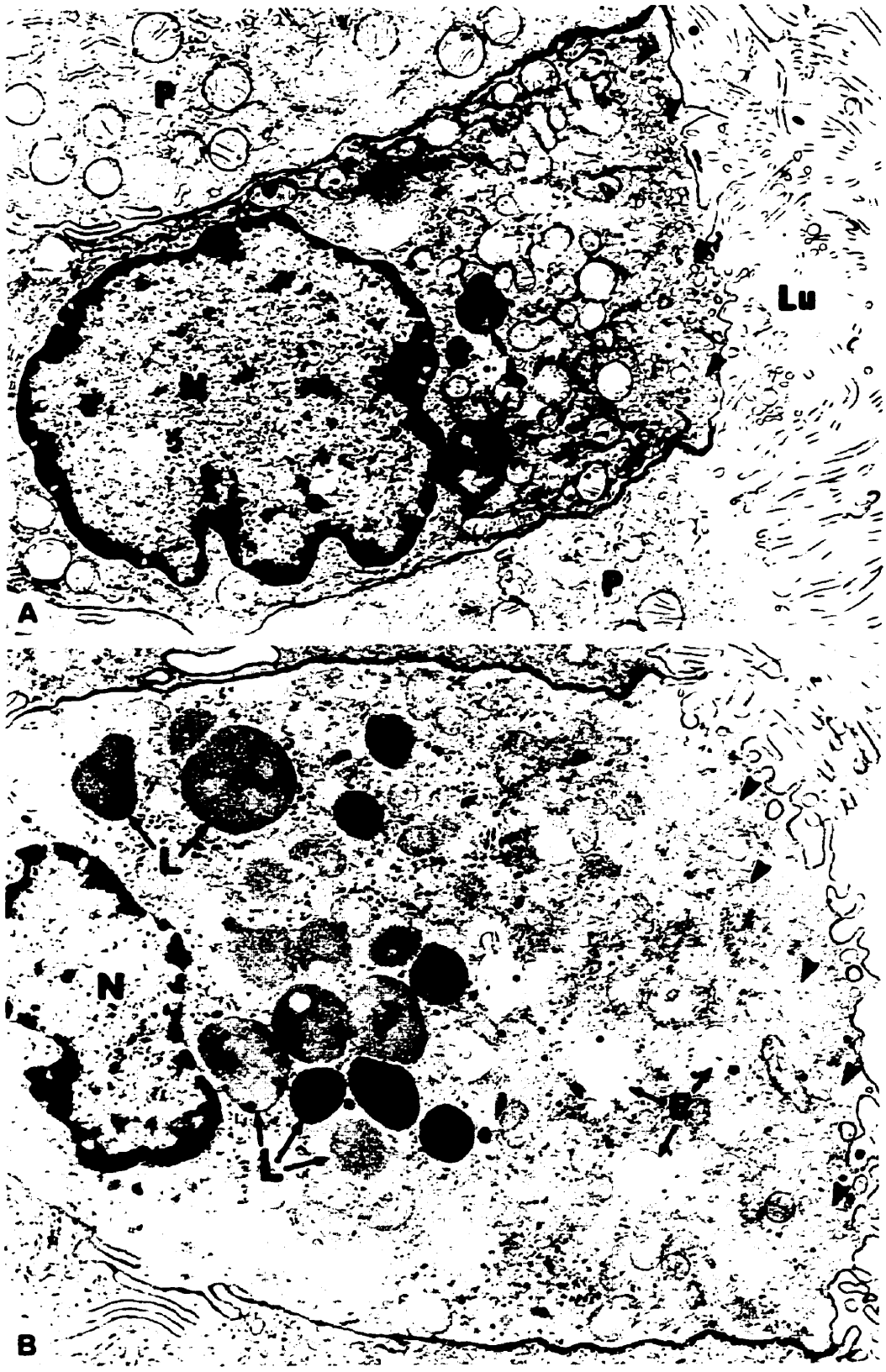
P
Lu
A
L
N
L
B

Fig. 10: Electron micrographs showing apical cytoplasmic blebs (B) of principal cells of the distal half of the middle vas deferens. In **(A)** and **(B)**, irregularly shaped blebs of cytoplasm project from the apical surface of principal cells into the lumen. They contain mainly filaments (f) and vesicles (large arrowheads) with microvilli (small arrowheads) extending from their surface. In **(C)**, a bleb of cytoplasm is far removed and apparently detached from the surface of a principal cell. In the lumen, numerous flattened and vesicular membranous profiles (arrows) are noted amongst the microvilli. JC, junctional complex. A: X 9,245; B: X 12,250; C: X 9,625.

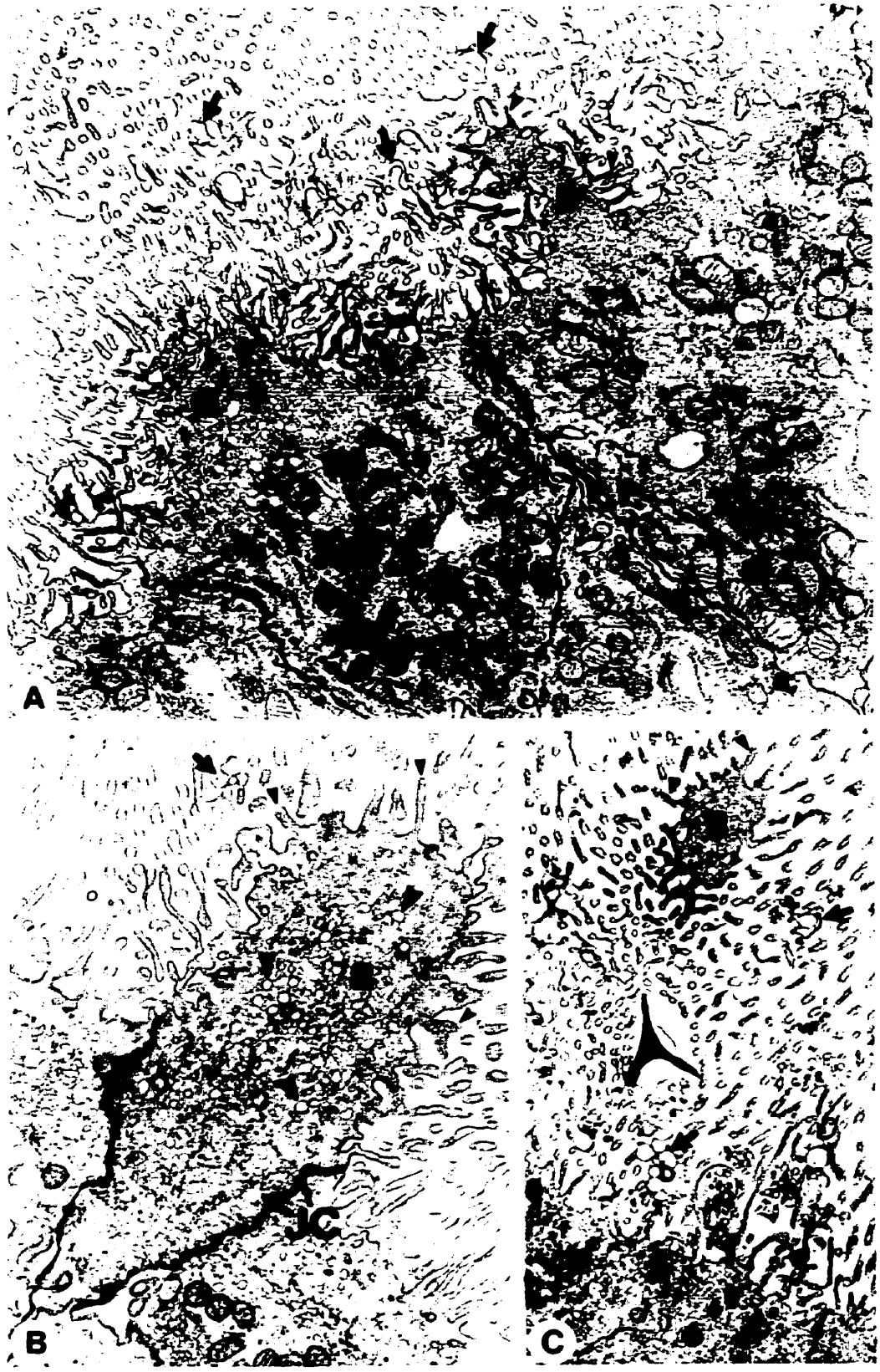
A
B
C
C

Fig. 11: Electron micrograph of the base of the epithelium of the distal region of the vas deferens. Closely apposed parallel sheets of smooth endoplasmic reticulum (sER) fill the basal area of principal cells, while a thin foot-like process (large arrow) attaches it to the basement membrane (BM). Conspicuous are the presence of large dilated intercellular spaces (asterisks) between adjacent principal cells and overlying basal cells (Ba). These spaces are variable in size and shape and extend for a considerable distance along the length of the epithelium. In the absence of close apposition of principal cells, the finger-like processes of principal cells are readily apparent and align up against each other (small arrows) or show focal contact points with each other (small arrowheads). The spaces contain empty looking spherical or irregularly shaped membranous profiles of various sizes (large arrowheads). These dilated spaces are often interrupted along their length by principal cells which show close points of approximation with each other (open arrows). Basal cells (Ba) contact the basement membrane but by being enveloped on their lateral surfaces by intercellular spaces, they reveal close contact points with each other (circles) and adjacent principal cells (squares). g, glycogen. X 10,750.

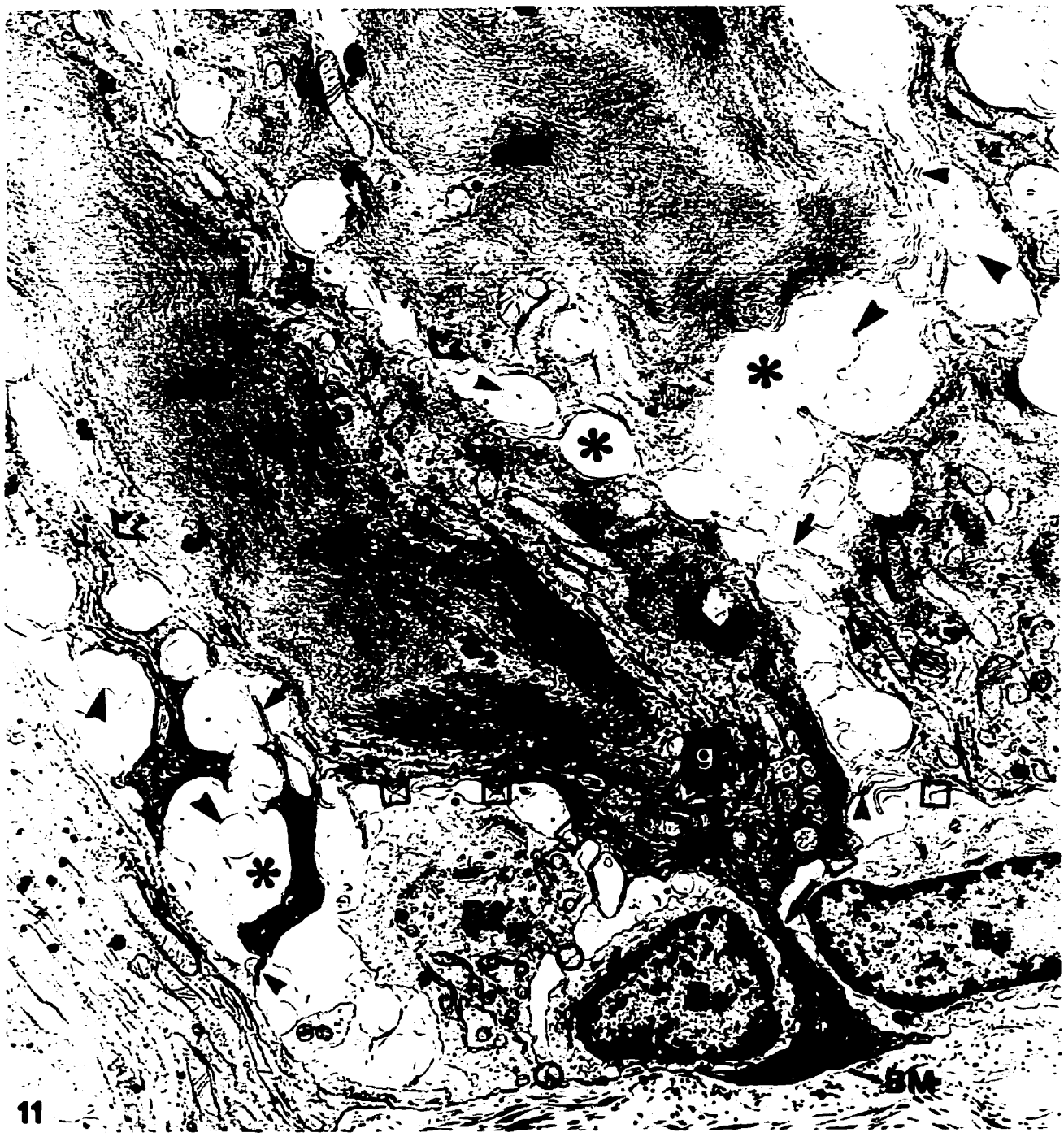

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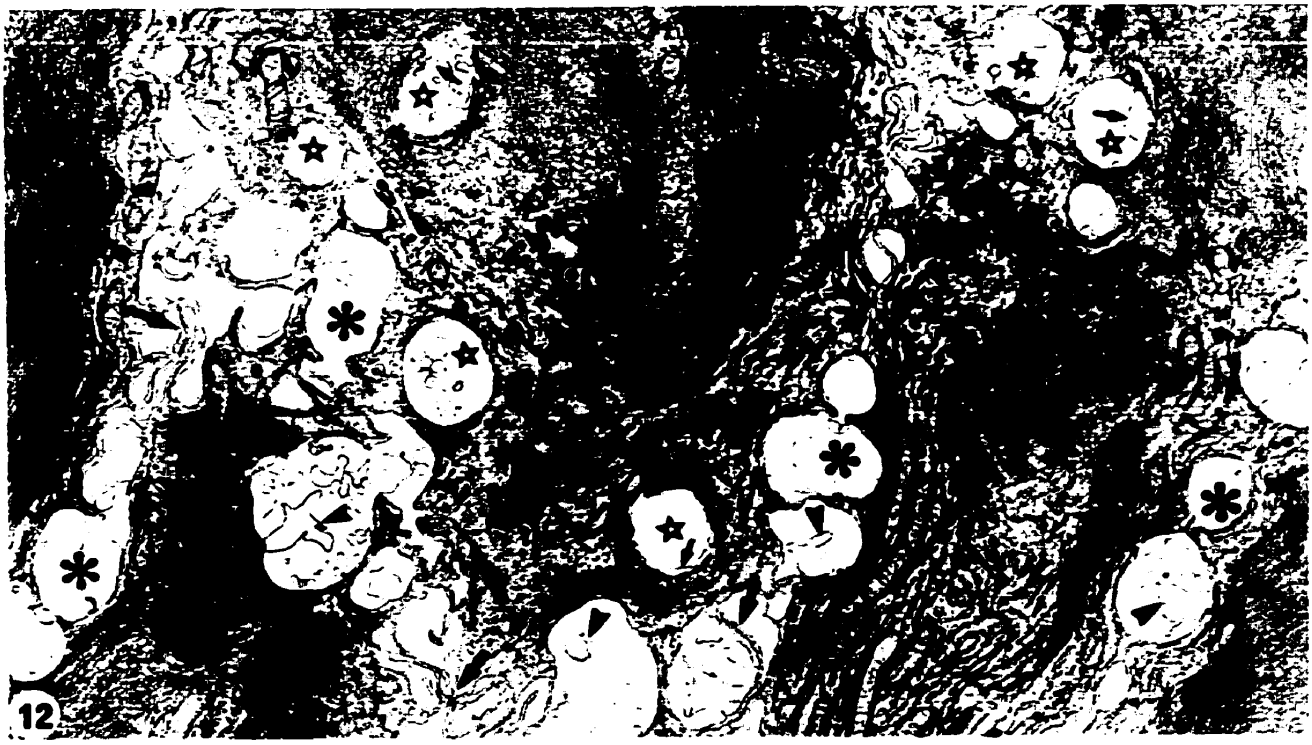
Fig. 12: Electron micrograph of the base of the epithelium of the distal vas deferens. The intercellular spaces (asterisks) between adjacent principal cells are dilated, irregular in appearance and contain membranous profiles of variable shapes and sizes (arrowheads). Along their length, principal cells show small or large focal points of contact with each other (open arrow). The finger-like processes of adjacent cells are readily visible and at times contact each other (large arrows). The cytoplasm of principal cells is filled with closely apposed parallel concentric flattened cisternae of smooth endoplasmic reticulum (sER). However, in areas of sER, large intracytoplasmic cavities (open stars) are surrounded by sER which at times is dilated and appears to peel off into the interior of the cavity (small arrows). These cavities are located deep in the cytoplasm or close to the lateral plasma membrane of the principal cell. g, glycogen. X 10,300.

Fig. 13: Electron micrograph of the base of the epithelium of the distal vas deferens. Finger-like processes of principal cells (large arrows) project into the dilated intercellular spaces (asterisks) between adjacent principal cells and basal cells (Ba). The cytoplasm of principal cells contains sER cisternae which in cross section form parallel flattened concentric rows (small arrowheads) or in face view appear as anastomotic networks (open arrows). Close to or removed from the intercellular spaces are intracytoplasmic cavities (open stars). The former may in another plane of section be connected to the lateral plasma membrane of the principal cell. Such cavities contain membranous profiles of different shapes and sizes which are also prominent in the intercellular space (large arrowheads). At one site, an intracytoplasmic cavity (solid star) surrounded by sER appears to be in the process of releasing its membranous contents into the intercellular space through an apparent disruption of the lateral plasma membrane (small arrow). X 22,500.



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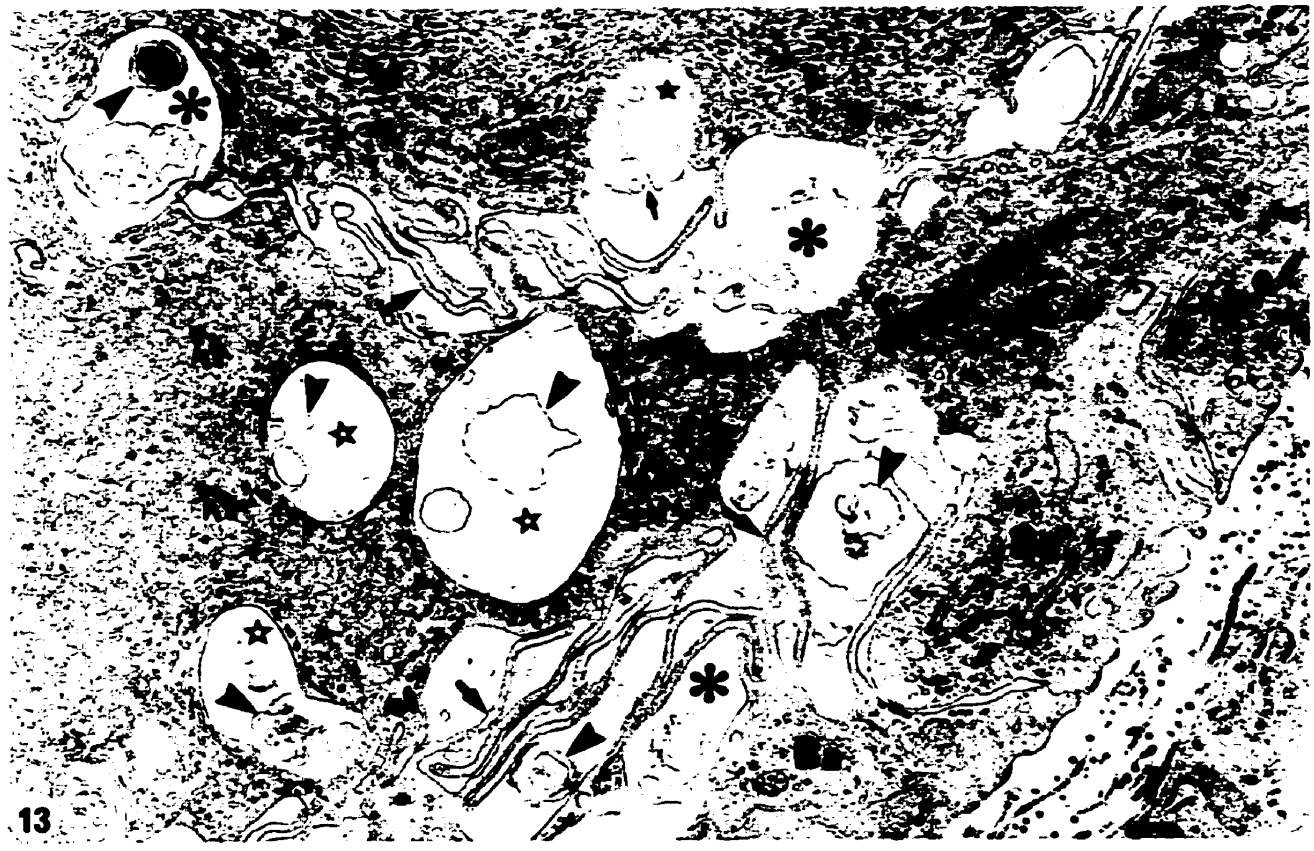

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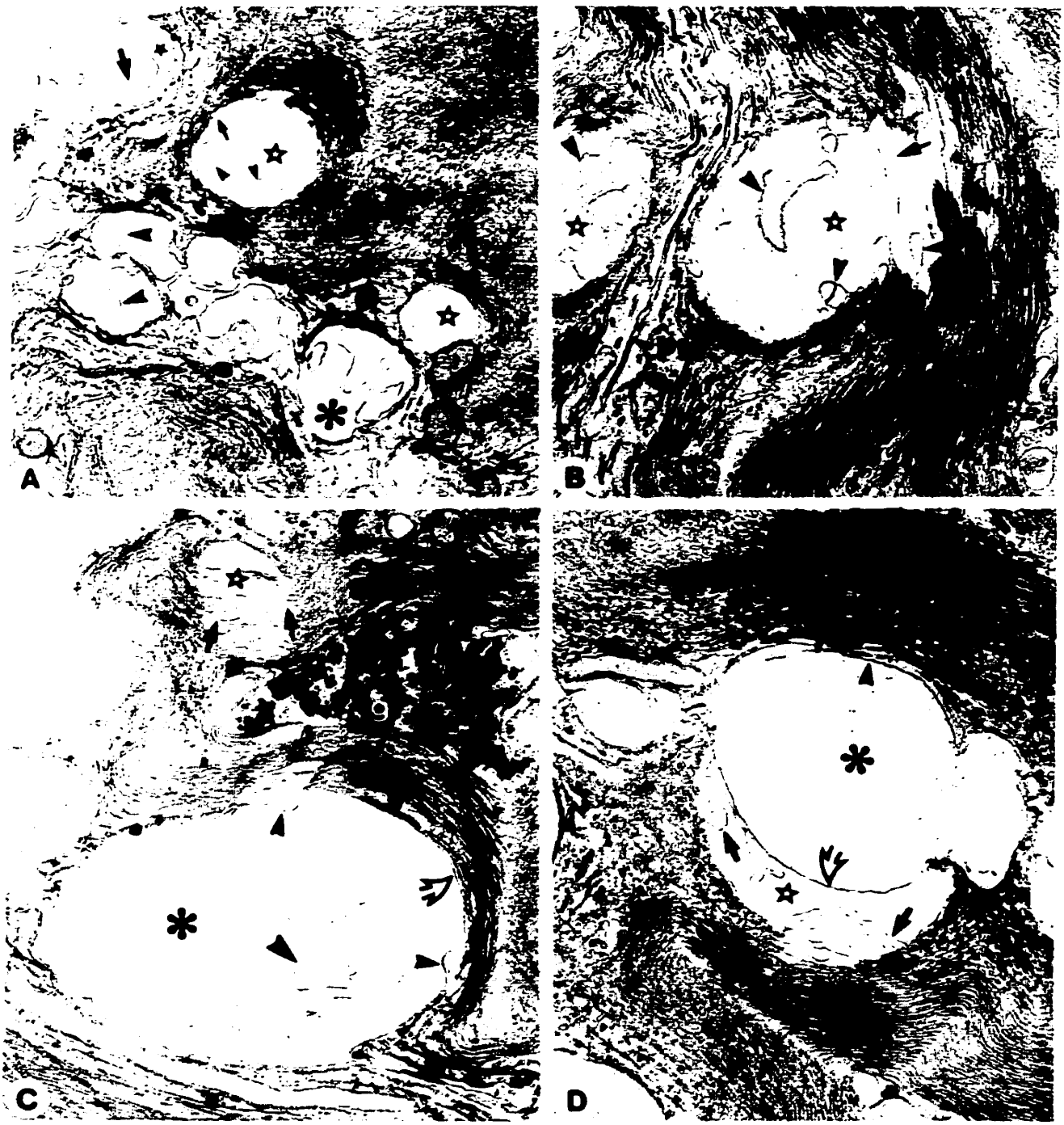
Fig. 14: Various images of intracytoplasmic cavities (open stars) in principal cells and dilated intercellular spaces (asterisks) at the base of the epithelium of the distal vas deferens. **(A)** An intracytoplasmic cavity is closely invested by concentric layers of sER which appear to peel off (small arrow) and fall into the interior of the cavity amongst other membranous profiles contained within (small arrowheads). The latter are similar to those present in the intercellular space (large arrowheads). At top left corner of the field, an intracytoplasmic cavity (solid star) appears to have fused with the lateral plasma membrane releasing its contents (large arrow) into the intercellular space. **(B)** Intracytoplasmic cavities contain membranous profiles (arrowheads) and are surrounded by sER cisternae, some of which show a dilated lumen (arrows). **(C)** An intracytoplasmic cavity is spanned by parallel layers of slightly dilated sER cisternae (arrows). The lateral plasma membrane of a principal cell (open arrow) bordering a dilated intercellular space is closely invested by layers of sER. Along its length it shows projections (small arrowheads) beneath which some sER cisternae appear slightly dilated. The intercellular space contains large irregularly shaped membranous profiles (large arrowhead). **(D)** The lateral plasma of a principal cell bordering one edge of a dilated intercellular space is closely invested with layers of flattened sER cisternae. Subjacent to the lateral plasma membrane forming its other edge (open arrow), is an intracytoplasmic cavity into which project several layers of slightly dilated sER cisternae (arrows), beneath which are multiple layers of concentric flattened layers of sER. g, glycogen; PM, lateral plasma membrane. A: X 14,700; B: X 23,100; C: X 19,950; D: X 16,800.


A
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C
D

Fig. 15: Lamina propria of the proximal **(A)** and **(B)** middle regions of the vas deferens. **(A)** Subjacent to the epithelium resting on a basement membrane (arrows) are several alternating myoid cell (My) and connective tissue layers (Ct). The myoid cells are filled with filaments (f), while collagen (arrowheads) occupies the connective tissue layers. **(B)** Immediately subjacent to the basement membrane of the epithelium (arrows) are several layers of fibrocytes (F) interspersed between connective tissue layers containing collagen fibers (Co), followed by myoid cell (My) layers. Ba, basal cell; Cap, capillary; N, nucleus of myoid cells; P, base of principal cells. A: X 10,300; B X 8,250.

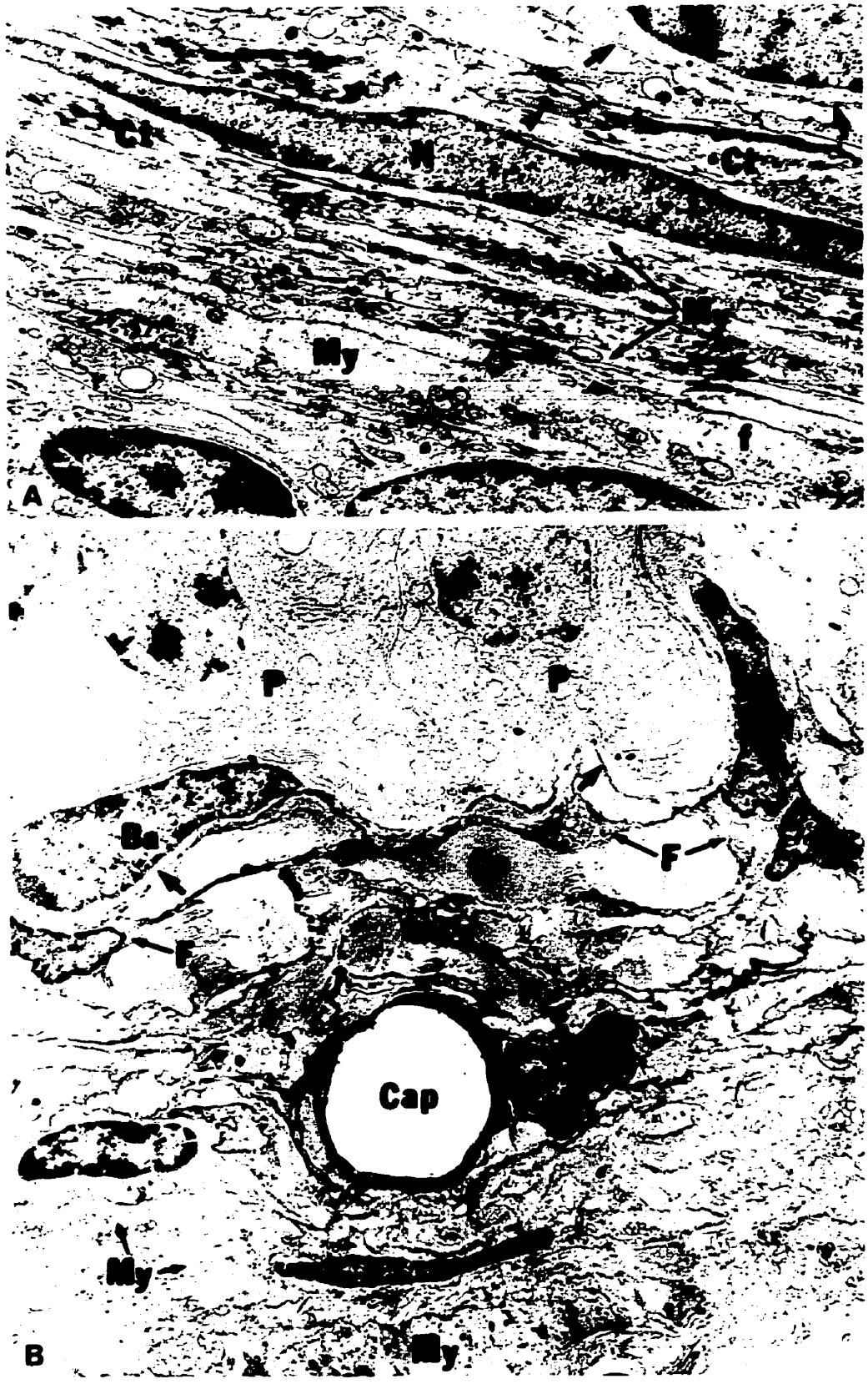
Ct
N
Ct
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Fig. 16: Electron micrograph of the lamina propria of the distal region of the vas deferens. Dilated intercellular spaces (asterisk) are noted between adjacent principal and basal (Ba) cells of the epithelium. Immediately subjacent to the basement membrane are multiple discontinuous layers of fibrocytes (F) interspersed between connective tissue layers containing collagen fibers (Co) and capillaries (solid stars), followed by large vascular channels (Va), and layers of myoid cells (My). X 3,800. Inset: Light micrograph of the distal vas deferens showing large vascular channels (Va) in the lamina propria. E, epithelium; Lu, lumen. X 218.

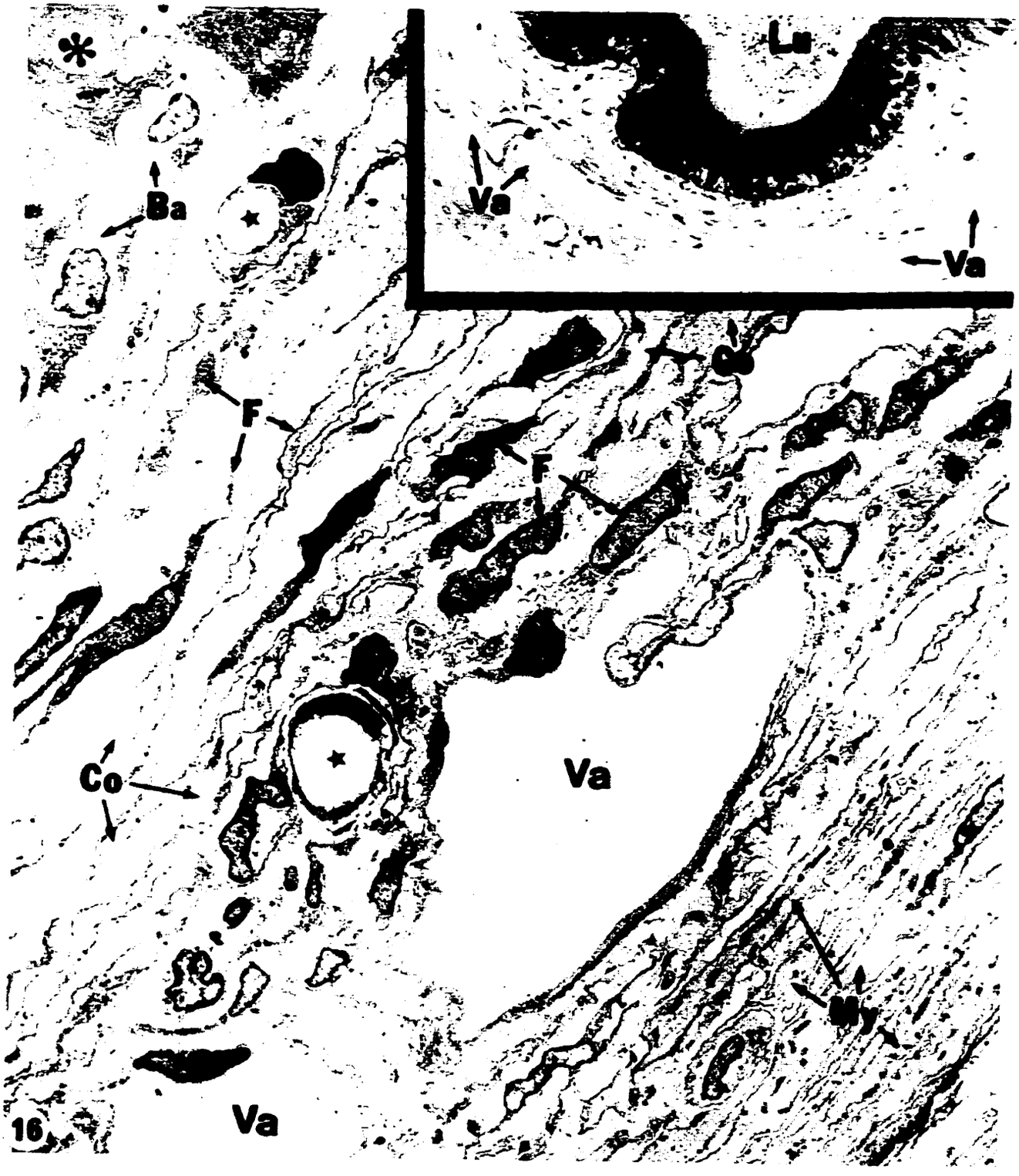
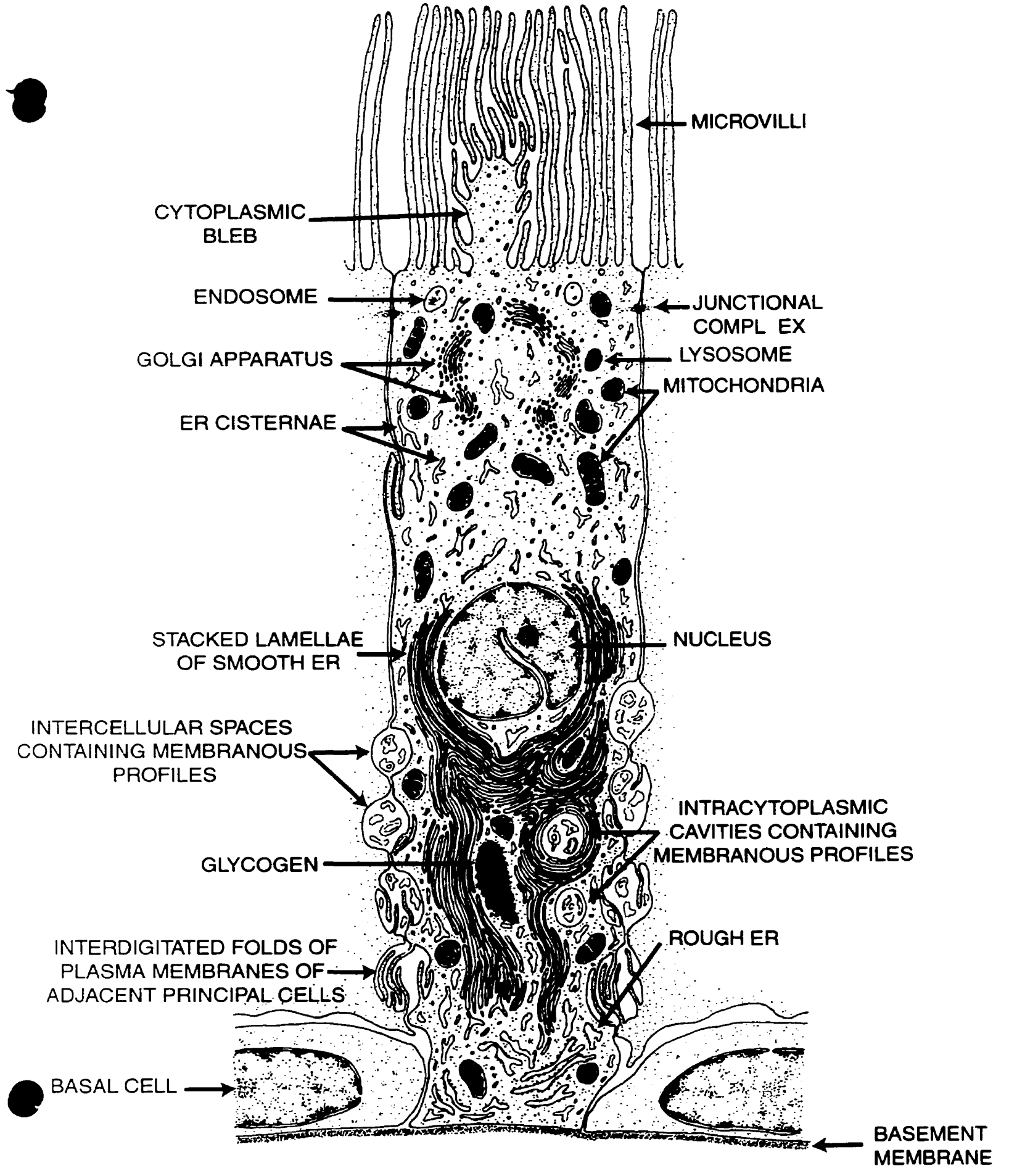

Lu
Va
Va
Ba
F
F
Co
Va
Va
16

Fig. 17: (A) Distal region of the vas deferens upon entry into the prostate gland immunostained with anti-AQP-1 antibody showing intense reactivity of the subepithelial vascular plexus (arrows). There is little reaction over the epithelium (E) of the duct or prostate gland (stars). X 218. **(B)** Corresponding section of the vas deferens lacking primary antibody as a control shows no reaction over the epithelium (E) of the duct, prostate gland (stars) or vascular plexus (arrows). X 218.

Fig. 18: Distal **(A)** and middle **(B, C)** regions of the vas deferens immunostained with anti 3β-HSD antibody. Reaction appears over the cytoplasm of principal cells (P). In **(A)** blebs of apical cytoplasm are noted (open arrows), one of which is well removed from the cell but still attached by a thin strand (arrow). In **(B),** numerous irregularly shaped blebs extend from the apical surface of principal cells (open arrows), while in **(C)** many blebs are noted free in the lumen (arrowheads). A reaction appears over these blebs. A: X 544, B: X 544; C: 218.


E
Lu
E
A
E
Lu
E
B
B
A
C

Fig. 19: Diagrammatic representation of a principal cell in the distal vas deferens. Emanating from the apical plasma membrane are tall microvilli. A cytoplasmic bleb is shown protruding from the apical surface into the lumen of the vas deferens. Various images suggest that such blebs detach from the surface of principal cells to be liberated into the lumen suggesting apocrine secretion. The apical/supranuclear cytoplasm contain numerous stacks of Golgi saccules along with tubular ER cisternae, while the infranuclear region contain stacked flattened sheets or lamellae of sER. Scattered amongst these sER elements are rough ER, mitochondria, and glycogen. Dilated intercellular spaces are noted between adjacent principal as well as basal cells. They contain membranous profiles of different shapes and sizes. In different areas of the sER aggregates, large intracytoplasmic cavities are present containing membranous profiles. The latter appears to be peeling off from the surrounding sER elements and are similar in appearance to those found in the intercellular spaces. Images of such cavities are noted close to the lateral plasma membrane suggesting release of membranous profiles into the intercellular space. The blebs and membranous profiles may represent a means of transporting steroids to the lumen and lamina propria, repectively.


MICROVILLI
CYTOPLASMIC BLEB
ENDOSOME
JUNCTIONAL COMPL EX
GOLGI APPARATUS
LYSOSOME
MITOCHONDRIA
ER CISTERNAE
STACKED LAMELLAE OF SMOOTH ER
NUCLEUS
INTERCELLULAR SPACES CONTAINING MEMBRANOUS PROFILES
INTRACYTOPLASMIC CAVITIES CONTAINING MEMBRANOUS PROFILES
GLYCOGEN
ROUGH ER
INTERDIGITATED FOLDS OF PLASMA MEMBRANES OF ADJACENT PRINCIPAL CELLS
BASAL CELL
BASEMENT MEMBRANE

2.6. DISCUSSION:

In the present study, we divided the vas deferens, identified as the straight tube emanating from the coiled tubules of the distal cauda epididymidis, into three equal portions referred to as proximal, middle, and distal regions, with the latter including a portion within the prostate gland. As noted by others (Niemi, 1965; Flickinger, 1973; Cooper and Hamilton, 1977; Hamilton and Cooper, 1978; Kennedy and Heidger, 1979), the principal cell structure varied between regions. Furthermore, only in the proximal region, several clear and narrow cells were noted, each with distinctive features. Basal cells resided in all regions. The focus of this discussion will be on the major cell type lining the vas which is the principal cell.

2.6.1. Apocrine secretion:

It is well recognized that principal cells of the vas deferens are active secretory cells containing large amounts of rER and a large Golgi apparatus (Flickinger, 1973; Hamilton, 1975; Hamilton and Cooper, 1978; Kennedy and Heidger, 1979). Differences in synthesis and secretion of proteins were noted in vitro between epithelial strips from the proximal and distal regions of the vas deferens (Wenstrom and Hamilton, 1984). However, over the years it has been noted that blebs or protrusions of cytoplasm devoid of most organelles emanated from the apical surface of principal cells of the rat vas deferens and were also seen free in the lumen (Niemi, 1965; Flickinger, 1973). Such blebs were also reported in the efferent ducts and various reproductive accessory organs from different species where they have been suggested to represent an apocrine type of secretion (Brandes, 1966; Dahl et al., 1973; Nicander et al., 1974; Riva et al., 1982; Pudney and Fawcett, 1984; Agrawal and Vanha-Perttula, 1988; Ilio and Hess, 1994; Renneberg et al., 1995). In the present study we noted apical blebs of principal cells predominantly in the middle region of the vas deferens where they were attached to the apical surface of principal cells or appeared free in the lumen (Figs. 10, 19). They lacked major organelles but contained few vesicular and tubular profiles. Due to structural integrity of principal cell organelles and intact junctional complexes, these blebs appear to represent a true structural feature of these cells and not a fixation artefact. Recently, a tissue specific major mouse vas deferens protein without a signal peptide was shown to be secreted via apical blebs in an apocrine manner (Manin et al., 1995). While the functional

significance of secretion involving a portion of the cell cytoplasm and plasma membrane is unclear, it raises four interesting questions. Firstly, how is the principal cell size maintained. Secondly, how are organelles segregated. Thirdly, what advantage does apocrine secretion have over merocrine secretion via secretory granules. Fourthly, how do the apical blebs detach from the cell surface and eventually break up and release their contents. Our results noted that the blebs contained 3β-HSD suggesting that they could provide steroids into the lumen for utilization by sperm (see section below). However, the functional roles of the blebs and their contents in relation to sperm in the lumen has yet to be fully determined.

2.6.2. Water transport:

In the middle and distal vas deferens, dilated intercellular spaces were noted between adjacent principal cells from the mid point to the base of the epithelium (Fig. 19). They also enveloped adjacent basal cells. Within these spaces, membranous profiles of different shapes and sizes were observed. In aggregates of the sER present in the infranuclear and basal cytoplasm of principal cells, empty looking intracytoplasmic cavities were noted. Such cavities either far removed or close to the lateral plasma membrane of principal cells, contained membranous profiles which appeared to represent sER elements peeling off from the aggregate (Fig. 19). Several images suggested that the membranous profiles of these cavities were being liberated into the dilated intercellular spaces. In fact, such profiles or cavities were not noted higher up in the lateral intercellular space or principal cell cytoplasm, respectively, nor were they found in the subjacent lamina propria indicating they did not come from the lumen or subepithelial areas. How this is accomplished is not clear, but it may be proposed that the lateral plasma membrane in areas adjacent to cavities may disrupt temporarily, resulting in release of membranous profiles into the dilated spaces. This mechanism is at present hypothetical.

The presence of dilated intercellular spaces has been noted in a variety of other locations such as the epithelial cells lining the efferent ducts, gall bladder, small and large intestine (Kaye et al., 1966; Pudney and Fawcett, 1984). In these tissues it has been reported that such spaces suggest the transport of fluid from the lumen to the underlying lamina propria. In the present study, intracytoplasmic cavities in principal cells close to or far

removed from the plasma membrane have an empty looking appearance suggesting a washing out of the cytosol. This may represent images of fluid passing through the cell at any given time point. Fluid accumulation in sER aggregates may be responsible for their disruption and subsequent release of sER elements into the dilated spaces. While we do not have solid data for the role of the sER membranous elements in the intercellular space, it is postulated at present that they may represent a means of steroid transport out of principal cells (see section below). Since steroids are soluble in membranes and hydrophobic, their exit from the cell may be facilitated by transport via the sER membranous profiles into the dilated spaces.

In the distal vas deferens large vascular channels were located separating the fibrocyte layers immediately beneath the basement membrane from the myoid cell layers. These channels have been reported previously (Kormano et al., 1972) and considered to be a venous plexus (Hamilton and Cooper, 1978). However, in the present study large irregularities and thinness of the endothelial wall suggest morphologically that these channels might be lymphatics. In the present study, we noted the localization of anti-AQP-1 antibodies within the endothelium lining these channels. Aquaporins are water channel proteins that play important roles in regulation of water transport across cellular membranes (Verkman et al., 1996). AQP-1 has been found in different sites in the kidney, choroid plexus, eye, gall bladder and its ducts and intestine, where it is involved in water transport across the epithelium (Sabolic et al., 1992; Neilson et al., 1993). In the vas deferens, Brown et al. (1993) noted a spotty reaction for anti-AQP-1 antibodies over the apical plasma membrane of epithelial cells of the ampulla region (Brown et al., 1993). Their use of frozen sections unlike our Bouin-fixed tissue may account for differences in sensitivity of the reaction, however, no mention was made of a reaction over vascular channels. However, AQP-1 has been noted to be present over the endothelium of lymphatic vessels and continuous capillaries in the small intestine (Nielson et al., 1993). Thus our present data suggest that water is transported from the lumen of the vas deferens through the epithelium to the dilated spaces and eventually to the underlying vascular channels. It has been demonstrated that the lumen of the proximal vas deferens is larger than that of the distal region (Niemi, 1965;

Hamilton and Cooper, 1978; Kennedy and Heidger, 1979). It might, therefore, be suggested that transport of water from the lumen in the distal vas deferens region would serve to concentrate sperm and eventually allow for more efficient interactions with secretions from the accessory glands during ejaculation. Our future studies involve electron microscope immunocytochemical analysis of AQP-1 to determine its subcellular localization and examination of other AQP antibodies.

2.6.3. Steroid synthesis:

It has long been recognized that principal cells of the middle and distal regions of the vas deferens contain an abundance of sER which has been suggested to have a steroid synthesizing function (Niemi, 1965; Flickinger, 1973; Hamilton, 1975; Kennedy and Heidger, 1979). In vitro studies revealed that a significant amount of labeled acetate is incorporated into both cholesterol and testosterone along the entire length of the vas deferens, and that testosterone production is 1.5 times greater in the vas than the caput epididymidis (Hamilton and Fawcett, 1970; Hamilton, 1971). This feature of the vas was attributed to the extensive whorls of sER which was reconstituted by administration of testosterone after castration (Hamilton et al., 1969). It has also been demonstrated that the vas metabolizes testosterone into several major steroids (Kumari et al., 1980; Seethalakshmi et al., 1982). Furthermore, demonstration of steroid synthesis in the vas deferens has also been performed by histochemical localization of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD in several species other than the rat (Tingari, 1973; Prakash and Moore, 1982).

In the present study, 3β-HSD was immunolocalized mainly within principal cells of the middle and distal vas deferens. Reaction was especially prominent in the infranuclear region of the cell corresponding to the area of extensive sER whorls noted in the electron microscope (Fig. 18). These data thus further support the idea that the vas deferens is involved in steroid synthesis. As suggested by others, the synthesis of steroids by the vas may ensure a constant pool of steroids that could be utilized by the sperm as they pass through the duct (Hamilton, 1971). The fact that the apical blebs, emanating from principal cells and appeared to be free in the lumen of the vas, were stained for 3β-HSD would suggest that synthesized steroids may reach the lumen via these structures. On the other hand, steroids

destined ultimately for the underlying lamina propria would be transported out of the principal cell via the membranous profiles into the dilated intercellular spaces. Once outside the principal cell, they could be released from the membranous profiles and be utilized in part by the myoid and smooth muscle cell layers, well known to be regulated by androgens (Syms et al., 1985; Longhurst, 1990). We are presently using electron microscope immunocytochemistry to resolve the subcellular localization of 3β-HSD in the vas deferens.

2.7. ACKNOWLEDGMENTS:

The authors wish to thank the excellent technical assistance of Jeannie Mui, Matilda Cheung and P.B. Mukhopadhyay. We greatly appreciate the drawing of Figure 19 prepared by Dr. Y. Clermont. The 3β-HSD antibody was kindly provided by Dr. Van Luu-Thé.

CHAPTER THREE:

Immunocytochemical Localization of the Ya, Yb1, Yc, Yf and Yo Subunits of Glutathione S-Transferases in the Cauda Epididymidis and Vas Deferens of Adult Rats

(Andonian S, Hermo L. *J Androl* 1999b)

3.1. ABSTRACT:

Glutathione S-transferases (GSTs) are dimeric proteins grouped into five classes based on the degree of amino acid homology of their subunits. They are involved in cellular detoxification by catalyzing the conjugation of reduced glutathione with various electrophilic substances. In the present study, the distribution of Ya and Yc subunits from the alpha family, Yb1 and Yo subunits of the mu class, and the Yf subunit of the pi class was examined with light microscope immunocytochemistry in Bouin-fixed, paraffin-embedded tissue of different regions of the cauda epididymidis and vas deferens. In the cauda, principal cells showed high levels of expression of Ya, Yc and Yo subunits, while in the vas deferens staining decreased to moderate levels for Ya and Yo and low levels for Yc. While Yf was maintained at low levels in principal cells of all cauda and vas deferens regions, Yb1 expression was more erratic, being checkerboard-like in the proximal vas deferens and showing moderate cytoplasmic but intense nuclear reactivity in all other regions. Basal cells in the cauda were intensely reactive for Yf, while in the vas deferens they became unreactive. Conversely, basal cells were unreactive for Ya in cauda and proximal vas deferens, while in the middle and distal vas deferens they became moderately reactive. In the case of Yb1 and Yo, some basal cells were reactive while others appeared unreactive in all cauda and vas deferens regions. Yc displayed both reactive and unreactive basal cells in the cauda regions, and while moderately reactive in the proximal vas deferens, they became intensely reactive in the middle and distal vas deferens. In summary, both principal and basal cells show varying degrees of GST expression in the different regions of the cauda and vas deferens, suggesting a complex, changing environment of substrates to which these cells are subjected. Furthermore, while expression often differs between principal and basal cells, the absence of reactivity of a given GST in one cell type is usually compensated for by expression in the other cell type in any given region of the cauda or vas deferens. Taken together the data suggest that ample protection from harmful circulating electrophiles can be provided for sperm during their storage in the cauda and vas deferens. In addition, since principal cells of the vas deferens are involved in steroid synthesis, the presence of GSTs in these cells may also serve to bind steroids or be involved in steroid isomerization.

3.2. INTRODUCTION:

Glutathione S-transferases (GSTs) are a family of soluble isozymes involved in cellular detoxification. They prevent the build up of potentially toxic substances by catalyzing the conjugation of reduced glutathione with various electrophilic substances. In addition to their main function in detoxification, they also function in steroid isomerization (Benson et al., 1977), glutathione peroxidation (Prohaska and Ganther, 1976), leukotriene C biosynthesis (Pemble et al., 1986), and can bind noncovalently to a number of nonsubstrate ligands including steroids (Ketley et al., 1975; Homma et al., 1986).

The soluble GSTs are dimeric proteins from a multigene family with five classes (alpha, mu, pi, theta, and sigma) grouped according to the amino acid homology of their subunits (Mannervik and Danielson, 1988; Buetler and Eaton, 1992; Daniel, 1993). The alpha gene family contains the Ya, Yc, and Yk subunits, the mu gene family consists of Yb1-4, Yn, and Yo subunits, while the pi gene family consists of Yf subunit. GSTs form homo- or heterodimers from subunits that are members of one gene family but not between gene families (Mantle et al., 1990). GSTs have similar molecular weights but differ with respect to isoelectric point and substrate specificities (Jakoby et al., 1976). Cells have evolved such a vast number of GSTs to protect themselves against a wide variety of potentially harmful substances that they can encounter.

Hales et al. (1980) have shown that the epididymis-vas deferens contains high GST activity, approximately 50% of that found in the liver on a per-mg of protein basis, and that there are a large number of transferases in the epididymis-vas deferens which are differentially localized along the length of this tissue. Furthermore, the activities of the GSTs in the epididymis have been shown to be androgen dependent and often region specific (Robaire and Hales, 1982). These enzymes have been suggested to protect the epididymis and maturing sperm from harmful electrophiles (Hales et al., 1980).

Recently, we examined with light and electron microscopic immunocytochemistry, the distribution of different GST subunits along the epididymis of the adult rat. Yf expression was distributed in a region specific manner in principal and basal cells, with principal cells showing a decrease in staining down the duct while basal cells became intensely reactive in the corpus and proximal cauda regions (Veri et al., 1993). In contrast, Yo expression in principal cells was found to increase in intensity from the initial segment to the cauda

epididymidis, while basal cells were unreactive throughout the epididymis (Veri et al., 1994). Like the Yf subunit, the Yc, Yb1, Ya, and Yb2 showed region specific expression in principal and basal cells with each often differing from the other (Papp et al., 1995). The absence of reactivity in one cell type of a given region was usually compensated for by the reactivity in the other cell type. The varied GST expression in these cells along the epididymis was thought to ensure that sperm would be protected from a wide variety of blood-borne electrophiles as they traversed the duct and during storage in the cauda epididymidis (Papp et al., 1995).

The vas deferens of the adult rat has been examined ultrastructurally and such studies have demonstrated that principal cells revealed marked structural differences in its proximal, middle, and distal regions reflective of diverse functional activities (Hamilton, 1975; Robaire and Hermo, 1988). In the proximal region, principal cells resembled those of the cauda epididymidis, while in the middle and distal regions, they contained an abundance of smooth endoplasmic reticulum suggesting steroid synthesis (Hamilton and Fawcett, 1970; Hamilton, 1971; Flickinger, 1973; Kennedy and Heidger, 1979; Andonian and Hermo, 1999a). In the epithelium, dilated intercellular spaces were noted between adjacent principal cells of the distal vas deferens suggestive of water transport serving to concentrate sperm in the lumen of the vas deferens (Brown et al., 1993; Andonian and Hermo, 1999a). The vas deferens also contains basal cells, but little is known about their functions in this tissue. In addition to principal and basal cells, the proximal vas deferens also contain few clear cells and occasional narrow cells.

While the expression of different GST's has been examined in the epididymis up to the proximal cauda region (Papp et al., 1995), little is known about their cell and region specific distribution in the middle and distal cauda epididymidis as well as the different regions of the vas deferens. The purpose of the present study was to immunolocalize Ya, Yc, Yb1, Yf, and Yo in principal and basal cells in each of the major subdivisions of the cauda and vas deferens and note differences and similarities in GST expression between regions and cell types. The role of GSTs will also be discussed in relation to sperm protection and steroid metabolism.

3.3. MATERIALS AND METHODS

3.3.1. Tissue Preparation for Light Microscopy:

Four adult male Sprague Dawley rats (350-450 g) obtained from Charles River Laboratories (St. Constant, QC) were anesthetized with an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON). The cauda epididymidis and vas deferens were fixed by perfusion through the abdominal aorta in an anterograde manner with Bouin's fixative for 10 minutes. After perfusion, the cauda and vas deferens were removed and immersed in Bouin's fixative for an additional 24 hours. Prior to immersion, the vas deferens was divided into proximal, middle, and distal regions (Andonian and Hermo, in press) so that the entire duct of each animal would fit into one paraffin block. The cauda epididymidis was cut along its long axis in such a way that each of its subdivisions, i.e. proximal, middle, and distal, would be included for examination. After fixation, the tissues were dehydrated and eventually embedded in paraffin. The vas deferens was embedded to allow sectioning along its long axis.

Paraffin sections, 5 mm thick, were deparaffinized in xylene and hydrated in a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished in 70% ethanol containing 1% (v/v) H_2O_2. Once hydrated, the tissue sections were incubated in a 300 mM glycine solution to block free aldehyde groups.

3.3.2. Immunoperoxidase staining:

Immunoperoxidase staining of tissue sections was carried out according to the procedure of Oko and Clermont (1989). Polyclonal antibodies reactive against Ya, Yb1, Yc, Yf, and Yo subunits of glutathione S-transferase were kindly provided by Dr. John Hayes (University of Edinburgh, Scotland). Their purification and specificity have been characterized in Hayes and Mantle (1986) and Hayes (1988).

Before immunostaining, tissue sections were blocked for 15 min with 10% goat serum in Tris-HCl-buffered saline (TBS). This and subsequent treatments were accomplished by placing 100 ml of a solution onto a coverslip and overturning the tissue face of the slide onto the drop, thus ensuring that the entire tissue was treated with minimal fluid (Oko and Clermont, 1989). Coverslips were removed by dipping the slides in TBS containing 1% Tween-20 (TWBS). Sections were then incubated in a 37°C humidified incubation chamber

for 1.5 hr with their respective polyclonal antibodies (all diluted 1/100 in TBS). After four 5 min washes in TWBS, sections were once again blocked with 10% goat serum in TBS. They were then incubated for 30 min (at 37°C) with goat anti-rabbit IgG conjugated to peroxidase (Sigma Chemical Co., St. Louis, MO) diluted 1:250 in TBS. This was followed by four 5-minute washes in TWBS.

The final reaction product was achieved by incubating the sections for 10 min in 500ml of TBS containing 0.03% H_2O_2, 0.1M imidazole, and 0.05% diaminobenzidine tetrahydrochloride (Sigma Chemical Co.), pH 7.4. Slides were then washed in distilled H_2O and counterstained with 0.1% methylene blue for 1 min. The tissue was dehydrated by passing slides through a graded ethanol series, after which the sections were immersed in xylene and mounted with Permount. Tissue sections incubated without the primary antibody were used as controls.

3.4. RESULTS:

Immunostaining with anti-Ya antibody revealed an intense reaction over the cytoplasm of principal cells of each region of the cauda epididymidis (Figs. 1 a, b) which shifted to a moderate reaction over these cells along the entire vas deferens (Figs. 1 c, d). Basal cells of the entire cauda region were unreactive (Figs. 1 a, b), and while unreactive in the proximal vas deferens, they became moderately reactive in the middle and distal vas deferens (Figs. 1 c, d). Narrow cells present only in the proximal vas were moderately reactive as were smooth muscle cells of the vas deferens (Fig. 1c). Clear cells were unreactive throughout the cauda epididymis (Figs. 1a,b) and proximal vas deferens (not shown).

In the cauda, while the cytoplasm of principal cells was moderately reactive, their nuclei were intensely reactive for anti-Yb1 antibody (Figs. 2 a, b). The same staining pattern was observed over principal cells of the vas deferens (Fig. 2d), except in its proximal region, where they showed a checkerboard staining pattern with the cells being intensely, moderately or weakly stained (Fig. 2 c). In all regions of the cauda and vas deferens, some basal cells were reactive, while others were unreactive (Figs. 2 a-d). Narrow cells of the proximal vas deferens were moderately reactive (not shown), while smooth muscle cells of the vas deferens were unreactive (Fig. 2 c). Clear cells of the cauda (Figs. 2a, b) and proximal vas deferens (not shown) were unreactive.

Immunostaining with anti-Yc antibody revealed intensely reactive principal cells throughout the cauda epididymidis (Figs. 3 a, b), which shifted from moderate to weak reactivity in the proximal and distal vas deferens, respectively (Figs. 3c, d). Basal cells were both reactive and unreactive in all cauda regions (Figs. 3a, b). However, while moderately reactive in the proximal vas, they were intensely reactive in the middle and distal vas deferens (Figs. 3c, d). Narrow cells were moderately reactive (Fig. 3c), while smooth muscle cells of the vas deferens were moderately reactive (Figs. 3c, d). Clear cells were unreactive throughout cauda epididymis (Figs. 3a, b) and proximal vas deferens (not shown).

Immunostaining for anti-Yf antibody revealed weakly reactive principal cells of the cauda and vas deferens regions (Figs. 4a-d). On the other hand, while basal cells were intensely reactive in all regions of the cauda (Figs. 4a, b), they became unreactive throughout the vas deferens (Figs. 4c, d). Narrow cells and smooth muscle cells of the vas deferens were

weakly reactive (Figs. 4c,d). Clear cells were unreactive throughout the cauda epididymidis (Figs. 4b) and proximal vas deferens (not shown).

Immunostaining for anti-Yo antibody revealed intense reactivity for principal cells of the cauda regions (Figs. 5a, b) and moderate reactivity in the vas deferens (Figs. 5c,d). Both reactive and unreactive basal cells were found throughout the cauda and vas deferens (Figs. 5a-d). Narrow cells were moderately reactive (not shown), as were smooth muscle cells of the vas deferens (Fig. 5c, d). Clear cells of the cauda epididymidis and proximal vas deferens were unreactive (not shown).

Sperm in the lumen of the cauda and vas deferens were consistently unreactive. Control experiments obtained by omitting the primary antibody showed no reaction for all of the GST subunits (not shown), and images were similar to controls published in earlier studies (Veri et al., 1993, 1994).

3.5. FIGURES, TABLES AND LEGENDS:

Fig. 1: Different regions of cauda epididymidis and vas deferens immunostained with anti-Ya antibody. Adjacent tubules of the middle **(a)** and distal **(b)** cauda regions show intense reactivity over the cytoplasm of principal cells (P), while clear (arrows) and basal (arrowheads) cells are unreactive. The cytoplasm of principal cells (P) of the proximal vas deferens **(c)** are moderately reactive as are narrow cells (curved arrow), while basal cells (arrowheads) appear unreactive. Smooth muscle cells (Sm) show moderate reactivity. Principal (P) and basal (small arrowheads) cells of the distal vas deferens **(d)** are moderately reactive. The lamina propria (Lp) is marked by capillaries (small arrows) and vascular channels (va). Note large empty-looking dilated intercellular spaces (stars) at mid and basal areas of the epithelium. Asterisk, sperm in the lumen; IT, intertubular space. X 544 for each.

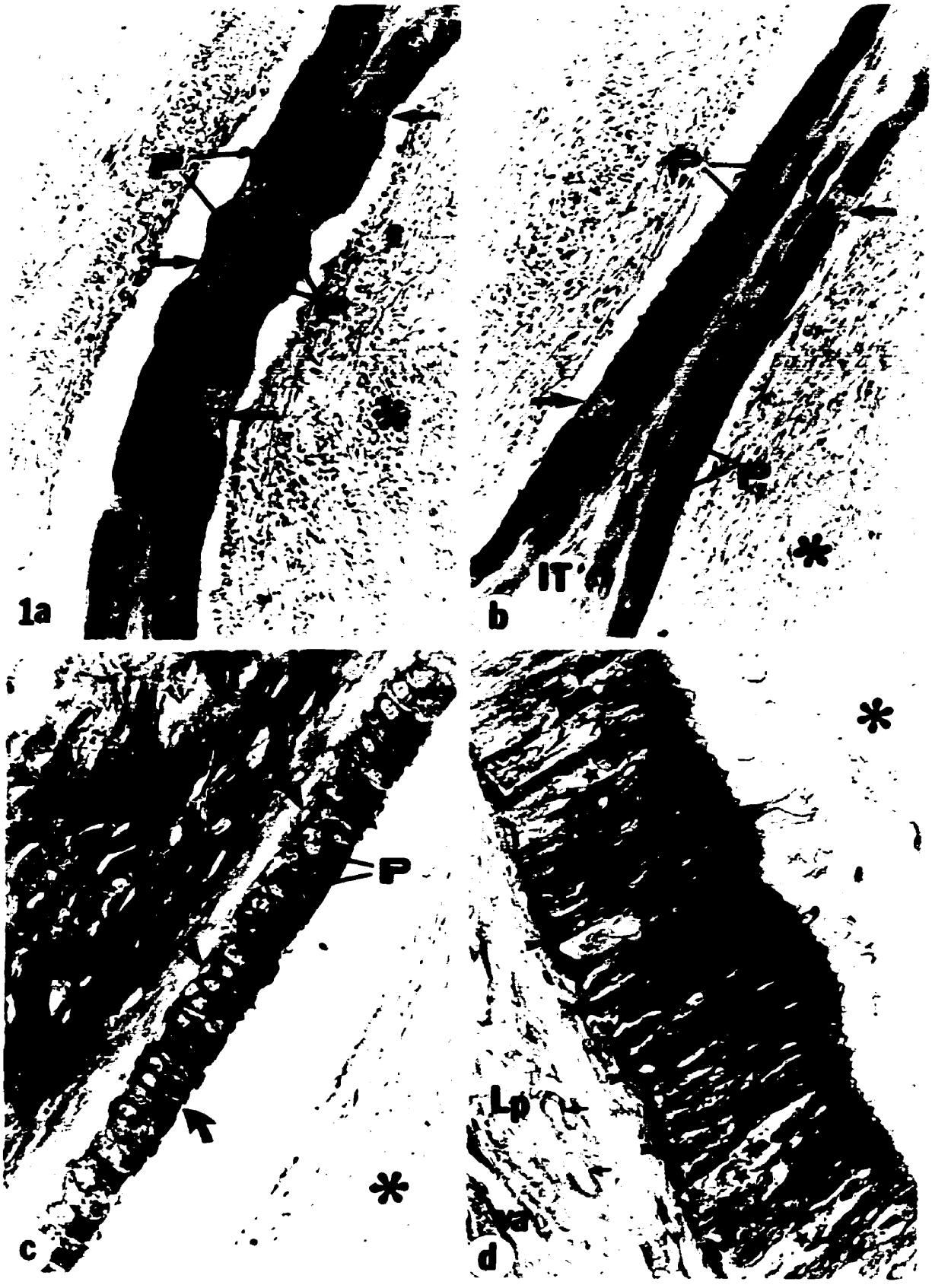

1a
b
IT
*
P
*
c
*
Lp
d

Fig. 2: Various regions of cauda epididymidis and vas deferens immunostained with anti-Yb1 antibody. Tubules of the middle **(a)** and distal **(b)** cauda regions show that the cytoplasm of principal cells (P) is moderately reactive, while their nuclei (n) are intensely reactive; clear cells (arrows) are unreactive. Some basal cells are reactive (small arrowheads), while others (large arrowhead) are unreactive. Principal cells (P) of the proximal vas deferens **(c)** show a checkerboard-like reaction, where cells are either intensely, moderately, or weakly reactive. Some basal cells are reactive (small arrowheads), while others are unreactive (large arrowhead). Smooth muscle cells (Sm) are unreactive. Principal cells (P) of the distal vas deferens **(d)** show moderate cytoplasmic but intense nuclear (n) reactivity. Some basal cells are reactive (small arrowheads), while others (large arrowheads) are not. The lamina propria (Lp) is marked by capillaries (small arrow). Note large empty-looking dilated intercellular spaces (stars). Asterisk, sperm in the lumen; IT, intertubular space. X 544 for each.


n
P
P
2a
P
*
*
P
FT
b
Sm
P
*
c
Lp
d

Fig. 3: Various regions of cauda epididymidis and vas deferens immunostained with anti-Yc antibody. Principal cells (P) of the middle **(a)** and distal **(b)** cauda regions are intensely reactive, while clear cells (arrows) are unreactive. Some basal cells (small arrowheads) are reactive, while others (large arrowheads) appear unreactive. Principal cells (P) of the proximal vas deferens **(c)** show moderate reactivity as do basal cells (small arrowheads). Smooth muscle (Sm) are moderately reactive. Principal cells (P) of the distal vas deferens **(d)** are weakly reactive, while basal cells (small arrowheads) are intensely reactive. The lamina propria (Lp) contains vascular channels (va). Note large empty-looking dilated intercellular spaces in the epithelium (stars). Smooth muscle cells (Sm) are moderately reactive. Asterisk, sperm in the lumen; IT, intertubular space. X 544 for each.

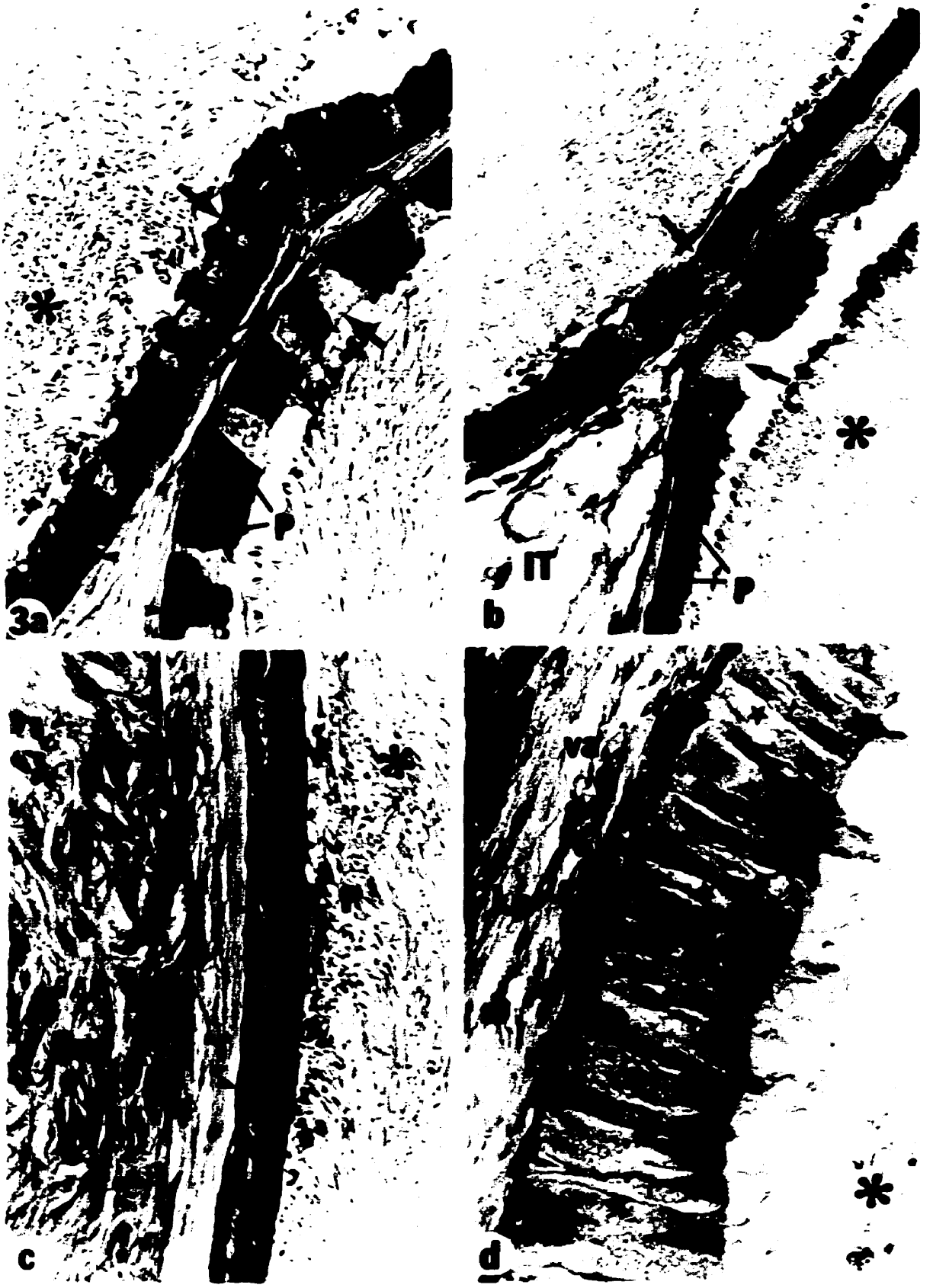

3a
b
c
d

Fig. 4: Middle cauda epididymis and various regions of vas deferens immunostained with anti-Yf antibody. Principal cells (P) of middle **(a, b)** cauda epididymis appear weakly reactive, while basal cells (small arrowheads) are intensely reactive. Clear cells (arrow) are unreactive. Principal (P) and narrow (curved arrow) cells of the proximal vas deferens **(c)** are weakly reactive, while basal (large arrowheads) cells appear unreactive. Smooth muscle cells (Sm) show weak reactivity. Principal cells (P) of the distal vas deferens **(d)** are weakly reactive, while basal cells are unreactive (large arrowheads). Note large empty-looking dilated intercellular spaces in the epithelium (stars). Asterisk, sperm in the lumen; IT, intertubular space; Lp, lamina propria. **(a)** X 870; **(b-d)** X 544.

c
d
a
b
T
P

Fig. 5: Different regions of cauda epididymidis and vas deferens immunostained with anti-Yo antibody. Principal cells (P) of the middle **(a)** and distal **(b)** cauda regions show intense reactivity. Some basal cells are reactive (small arrowheads), while few are unreactive. Principal cells (P) of the proximal vas deferens **(c)** show moderate reactivity. Some basal cells (large arrowheads) are unreactive while few are reactive. Smooth muscle cells (Sm) show moderate reactivity. Note moderately stained irregular membranous profiles (open arrow) in the lumen. Principal cells (P) of the middle vas deferens **(d)** are moderately stained, while some basal cells (small arrowheads) are weakly reactive. Note large empty-looking dilated intercellular spaces in the epithelium (stars). Smooth muscle cells (Sm) show weak reactivity. Asterisk, sperm in the lumen; IT, intertubular space; Lu, lumen; Lp, lamina propria. X 544 for each.

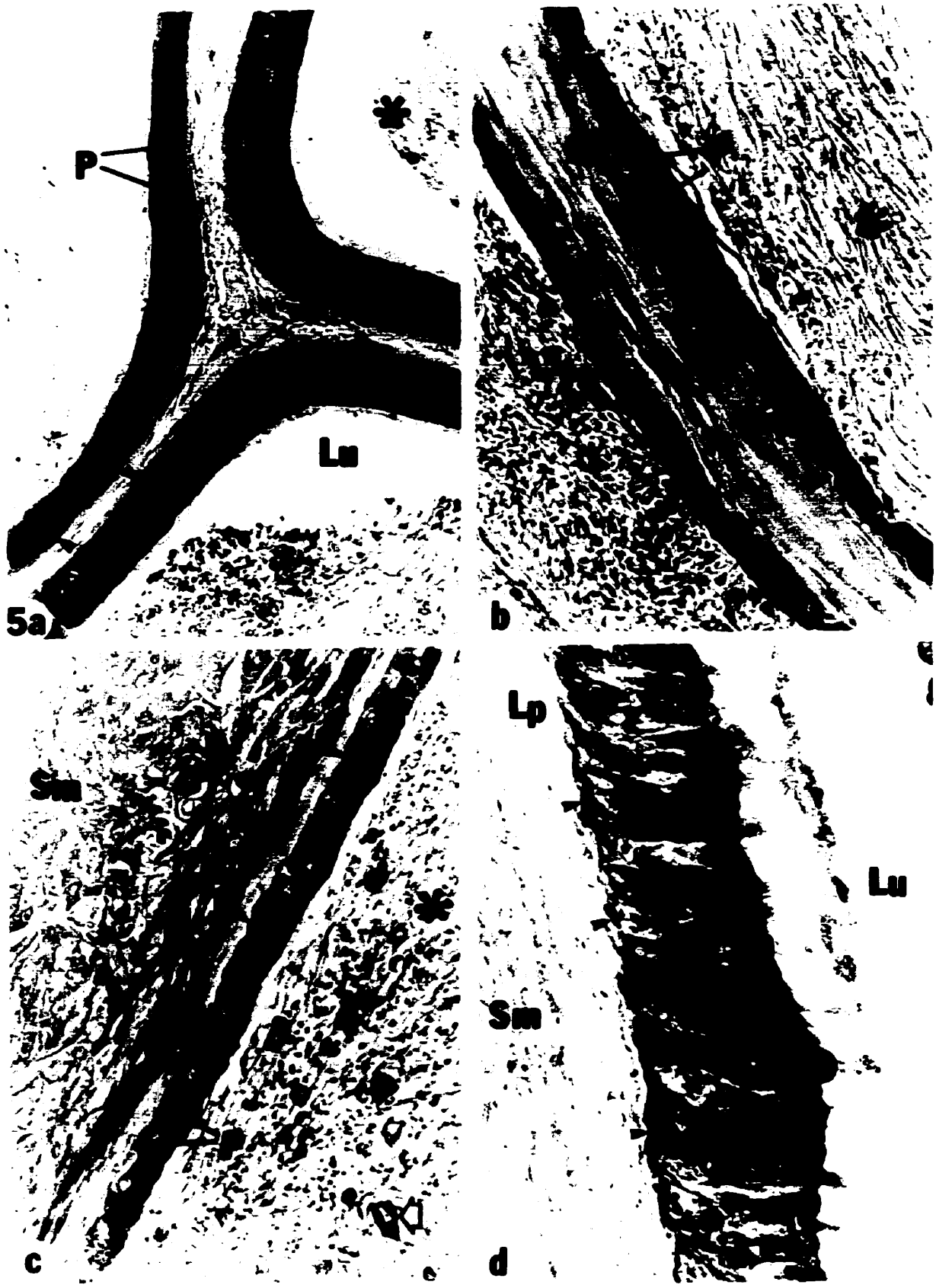

P
Lu
5a
b
Sm
c
Lp
Lu
Sm
d

Fig. 6: Diagrammatic representation of the staining pattern of the cauda epididymidis, and proximal (PVD), middle (MVD), and distal (DVD) vas deferens. Principal cells are represented with microvilli and show a progressive increase in size from cauda to DVD. Clear cells, present only in cauda and PVD are represented without microvilli and are consistently unreactive. Basal cells, found throughout, are represented as hemispherical cells. In the cauda, cytoplasm of principal cells shows high levels of expression of **Ya** subunit, while the nucleus is moderately reactive. In PVD, the cytoplasm of principal cells is moderately reactive with weaker reactivity in the nucleus. In MVD and DVD, principal cells are moderately reactive. Basal cells in the cauda and PVD are unreactive, while in the MVD and DVD they are moderately reactive. **Yb1** expression in principal cells, shows moderate cytoplasmic but intense nuclear reactivity in all regions except PVD, where a checkerboard pattern is evident. In all regions, basal cells are either reactive or unreactive. **Yc** expression in principal cells, shows intense reactivity in cauda, moderate reactivity in PVD, and weak reactivity in MVD and DVD. Basal cells are reactive and unreactive in cauda, moderately reactive in PVD, and intensely reactive in MVD and DVD. **Yf** expression in principal cells shows weak reactivity in all regions, while basal cells are unreactive except for the cauda where they are intensely reactive. **Yo** expression in principal cells, shows moderate reactivity in all regions except the cauda where they are intensely reactive. Basal cells are reactive and unreactive in all regions. Narrow cells, found only in PVD and represented as goblet shaped cells without microvilli, show moderate reactivity for all subunits except for Yf which is weak.

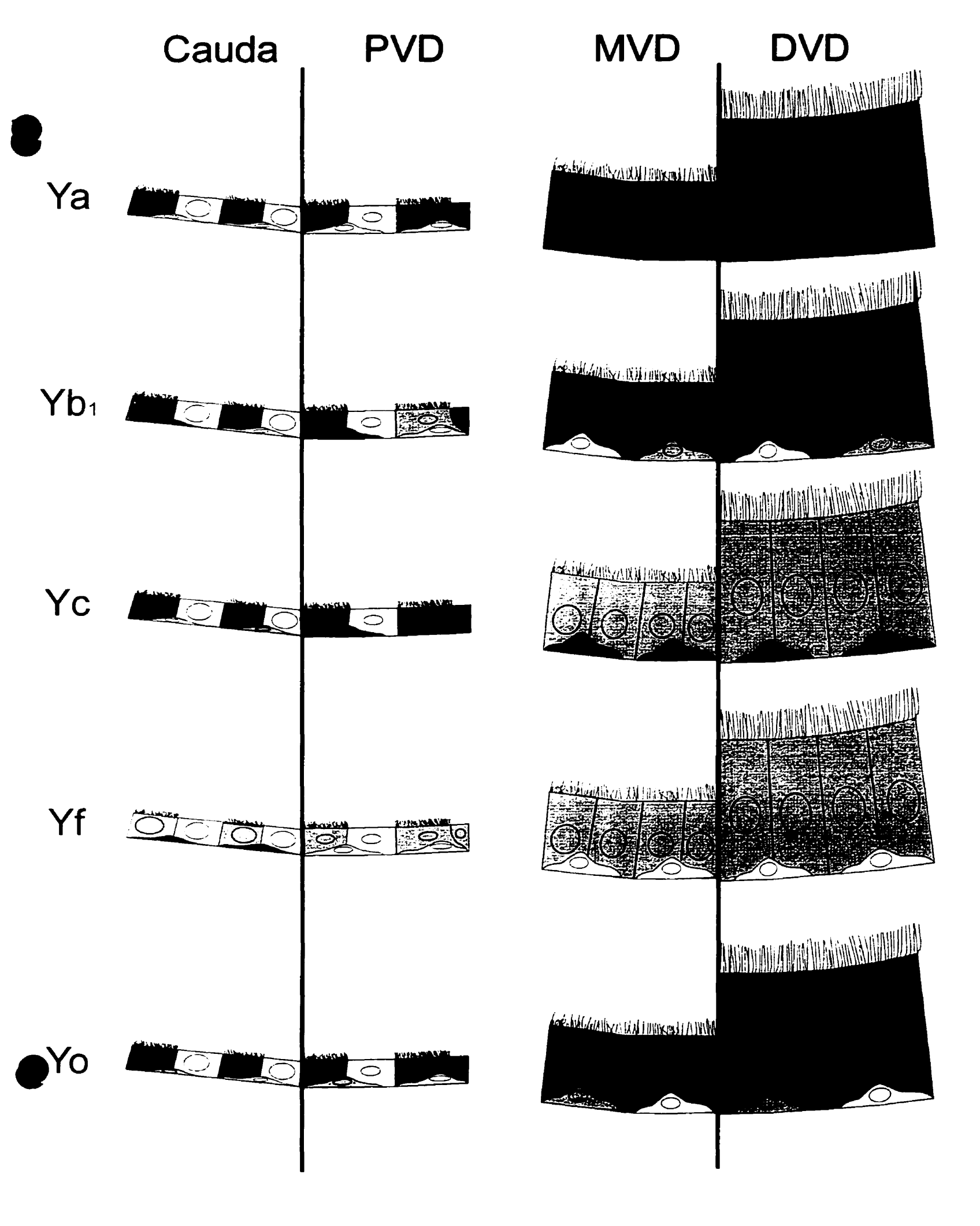

Cauda
PVD
MVD
DVD
Ya
Yb1
Yc
Yf
Yo

Table 1: Degree of immunostaining of different anti-GST antibodies over epithelial cells in different regions of the cauda epididymidis and vas deferens.

Region	Antibody / Cell Type	Ya	Yb1	Yc	Yf	Yo
PC[1]	Principal	+++[2]	++ (N)[3]	+++	+	+++
	Basal	-	+/-[4]	+/-	+++	+/-
MC	Principal	+++	++ (N)	+++	+	+++
	Basal	-	+/-	+/-	+++	+/-
DC	Principal	+++	++ (N)	+++	+	+++
	Basal	-	+/-	+/-	+++	+/-
PVD	Principal	++	(CB)[5]	++	+	++
	Basal	-	+/-	++	-	+/-
MVD	Principal	++	++ (N)	+	+	++
	Basal	++	+/-	+++	-	+/-
DVD	Principal	++	++ (N)	+	+	++
	Basal	++	+/-	+++	-	+/-

[1] PC, MC, DC, PVD, MVD, and DVD correspond to the proximal, middle, and distal cauda epididymal regions, and the proximal, middle, and distal regions of the vas deferens, respectively.

[2] The number of plus signs (+) is directly proportional to the strength of the reaction, while the minus sign (-) indicates the absence of reaction (+++, ++, and + correspond to intense, moderate, and weak reactivity, respectively).

[3] Nuclei (N) of the principal cells are more intensely stained than their cytoplasm.

[4] +/- indicates that while some basal cells are reactive, others are unreactive.

[5] CB indicates checkerboard reaction.

3.6. DISCUSSION:

The vas deferens is a tissue which has not been examined in much detail and is usually considered as simply a conduit for sperm during ejaculation. However, it has been noted that humans do not have a bulbous cauda region, and that a large portion of the proximal vas serves as a storage site for sperm (Turner, 1995). In many species, the low temperature of the cauda region facilitates sperm storage by enhancing oxygen availability to cells (Djakiew and Cardullo, 1986). However, sperm, while stored in this region, have been shown to be susceptible to high oxygen species as they contain high levels of polyunsaturated phospholipids in their membranes (Jones and Mann, 1977; Alvarez and Storey, 1989). As a result sperm must be protected as these oxygen species can result in lipid peroxidation of their membranes, membrane fragility and impaired fertility (Jones and Mann, 1977; Alvarez and Storey, 1989). The cauda epididymidis, therefore, contains various antioxidant molecules that prevent oxygen radical injury (Palladino and Hinton, 1995). In addition, the proximal cauda region has been shown to express various GSTs (Papp et al., 1995). However, a cell and region specific distribution of GSTs in the middle and distal cauda regions has yet to be performed. In addition, GST expression in the vas deferens, where sperm are also stored, has not been done.

3.6.1. Principal Cells:

Principal cells, the major epithelial cell type lining the vas deferens, secrete various proteins into the lumen, some of which associate with sperm (Hamilton, 1975; Hamilton and Cooper, 1978; Robaire and Hermo, 1988). Endocytosis also occurs whereby proteins can be removed from the lumen to be degraded by lysosomes of principal cells or transcytosed to the lateral intercellular space (Hermo and DeMelo, 1987). These cells also appear to be involved in apocrine secretion, synthesis of steroids and the removal of water from the lumen to the lateral intercellular spaces and underlying lamina propria (Niemi, 1965; Hamilton and Fawcett, 1970; Hamilton, 1971; Agrawal and Vanha-Perttula, 1988; Brown et al., 1993; Renneberg et al., 1995; Andonian and Hermo, 1999a). Thus, principal cells of the vas deferens have important roles to play in modifying the luminal environment in which sperm are stored. However, little is known about the expression of GSTs in principal cells and the role they play in this region.

In the present study, principal cells showed a variable pattern of expression for each

antibody, with expression of each GST often changing from one region of the cauda or vas deferens to the next (Table 1, Fig. 6). In the cauda region, principal cells showed high levels of expression of Ya, Yc and Yo subunits, while in the vas deferens staining decreased to moderate levels for Ya and Yo and low levels for Yc, suggesting an important role for these enzymes in the cauda and vas deferens where sperm are stored (Table 1). In comparison to principal cells in other epididymal regions, Papp et al. (1995) also showed a variable staining pattern for Ya, Yc, and Yo. However, in each case there was a tendency for expression to increase moving down the duct suggesting important roles for these proteins in the corpus and proximal cauda regions where sperm are stored (Papp et al., 1995). In the present study, Yf was weakly expressed in principal cells of the cauda and vas deferens regions, suggesting an insignificant role for Yf in these regions. Similarly, Yf was weakly expressed in principal cells of the the corpus and proximal cauda regions (Veri et al., 1993; Papp et al., 1995).

In the present study, the anti-Yb1 antibody showed moderate cytoplasmic, but intense nuclear reactivity from the cauda epididymidis to the distal vas deferens, except in the proximal vas deferens where principal cells showed a checkerboard staining pattern (Table 1, Fig. 6), suggesting an important role for Yb1 in these regions. The intense nuclear reaction over principal cells was also noted throughout most of the epididymis with Yb1 (Papp et al., 1995). It has been demonstrated that Yb1 proteins exist in a soluble form in both the nucleus and cytoplasm, however, they also exist in a bound form in the nucleus where they bind to DNA (Bennett et al., 1986; Hayes and Mantle, 1986; Ketterer et al., 1990). It has also been shown that Yb1 in carcinoma cells migrates into the nucleus, providing further evidence for their strong nuclear affinity (Bennett et al., 1986; Bennett and Yeoman, 1987). In the cauda and vas deferens, the Yb1 protein may protect DNA and RNA from harmful electrophiles that may be encountered by principal cells.

The checkerboard staining pattern noted for Yb1 protein in the proximal vas deferens has also been shown for principal cells in the distal caput epididymidis with Yb1 and Yf (Veri et al., 1993; Papp et al., 1995). It has been suggested that such a staining pattern may represent principal cells that are out of synchrony with each other with respect to synthesis of these proteins or that they show differing degrees of response to regulatory factors (Papp et al., 1995). Such a staining pattern has also been shown to occur for several secretory

proteins and be localized in different epididymal regions (Hermo et al., 1991, 1992; Rankin et al., 1992) indicating that such a pattern is a prominent feature with regard to synthesis of a variety of different proteins.

3.6.2. Basal Cells:

Basal cells are prominent in the epithelium of the rat vas deferens, but their functions are poorly understood. In the epididymis, basal cells expressed Yf at high levels in the corpus and proximal cauda regions, and the intensity of the reaction was such that it revealed that these cells possessed extensive attenuated processes that formed a discontinuous mesh-like network around the epididymal tubules (Veri et al., 1993). Basal cells also express superoxide dismutase (Nonogaki et al., 1992), and other GSTs have also been localized within epididymal basal cells often in a region-specific manner (Papp et al., 1995). The preferential localization of Yf and Yc subunits in the proximal caudal region suggested that they may protect sperm during their storage in this region (Papp et al., 1995).

In the present study, basal cells also showed a variable and often region specific expression for the various GSTs examined. Changes were also noted between the different regions of the cauda and vas deferens (Table 1, Fig. 6). In the cauda, basal cells showed intense expression for YF, but became unreactive in the vas deferens. In contrast, these cells showed no expression for Ya in the cauda and proximal vas deferens, but became moderately reactive in the middle and distal vas deferens (Table 1, Fig. 6). In the case of Yo and Yb1, both reactive and unreactive basal cells were noted in all cauda and vas deferens regions (Table 1). While in the cauda both reactive and unreactive basal cells were noted for Yc, in the vas deferens these cells showed a progressive increase in expression from moderate to high levels in the distal vas deferens (Table 1). The changing scenario of GST expression in basal cells suggests that they encounter different substrates in the different regions of the cauda and vas deferens. In addition, ample GSTs expression in basal cells could provide protection for sperm from harmful circulating electrophiles while stored in the cauda and vas deferens.

In the case of Yb1, Yc and Yo proteins, both reactive and unreactive basal cells were noted in various regions of the cauda and vas deferens suggesting that these cells are not in synchrony with each other with respect to the synthesis of these different GSTs, that they are under the control of different regulatory mechanisms or that some basal cells do not express

a given GST. It would be of interest eventually to determine whether the same or different basal cell expresses each of these three GST proteins by serial section immunocytochemistry, in situ hybridization or double and triple labeling using confocal microscopy.

3.6.3. Functional Significance:

In comparing the reactivity of GSTs in principal and basal cells, one is struck by the finding that the absence of a given GST from one cell type is usually compensated for by its weak to intense expression in the other cell type in any given region (Table 1, Fig. 6). Furthermore, while reactivity varies between cell types in given regions, at no point along the entire cauda or vas deferens are the various GSTs absent from both cell types. Noteworthy also is the finding that members within the same family (Ya, Yc) and (Yo, Yb1) do not always show the same levels of reactivity for principal or basal cells in a given region suggesting they carry out different functions (Table 1, Fig. 6). It is known that specificities of GSTs toward electrophilic substrates is broad and overlapping yet distinctive for each form of the enzyme. The need for such an array of GSTs may arise from the large variety of potentially harmful circulating substances that principal and basal cells encounter during their storage and transport in these regions.

In the vas deferens, principal cells have been documented to be involved in steroid synthesis (Hamilton and Fawcett, 1970; Hamilton, 1971; Andonian and Hermo, 1999a). It is well documented that GSTs function in binding steroids and in steroid isomerization. Several steroids were found to preferentially interact with Yb forms of GST due to their high binding affinity, and it was suggested that these subunits have the potential to function in metabolism, transport and perhaps even action of steroid hormones (Homma et al., 1986). The latter investigators also suggested that the Yb forms of GST could function alternatively as a safeguard to prevent undesired effects of steroid hormones. In the present study, Yb1 was localized within principal cells of the entire vas deferens suggesting that it may play a role involving steroids. In addition, in the rat, steroid isomerase activity has been shown to be associated principally with GST B, corresponding to Ya and Yc subunits (Benson et al., 1977), and in the present study, these subunits were expressed within principal cells of the entire vas deferens.

In the present study, clear cells of the entire cauda region and proximal vas deferens did not express any of the GSTs examined, a finding that is consistent with their unreactivity

throughout the entire epididymis (Papp et al., 1995). Narrow cells present only in the proximal vas deferens showed expression at moderate levels for all GSTs, except for Yf which was weakly expressed (Table 1, Fig. 6). Little is known of the functions of these cells, although the present data suggest that they play a role in ridding of harmful electrophiles. In addition, smooth muscle cells of the vas deferens were noted to express Ya, Yc, Yf and Yo suggesting that they may also play a role in the disposal of potentially harmful electrophiles.

In summary, principal and basal cells show varying degrees of expression of different GSTs, suggesting a changing environment to potentially harmful electrophiles that these cells encounter along the cauda epididymidis and vas deferens. While these GSTs may provide protection for sperm during their storage in these tissues, they may also be involved in steroid binding and steroid isomerization in principal cells of the vas deferens.

3.7. ACKNOWLEDGEMENTS:

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CHAPTER FOUR:

Cell and region specific localization of lysosomal and secretory proteins and endocytic receptors in epithelial cells of the Cauda Epididymidis and Vas Deferens of the Adult Rat

(Andonian S, Hermo L. *J Androl* 1999c)

4.1. ABSTRACT:

The epithelial cells lining the cauda epididymidis and vas deferens are active in endocytosis and have an abundance of lysosomes and a well characterized secretory apparatus. However, little is known about the nature of lysosomal proteins contained within lysosomes, the types of receptors on the cell surface, and the types of proteins secreted by these cells. In the present study, cathepsins A, D, B, and sulfated glycoprotein (SGP)-1, well characterized lysosomal proteins, as well as SGP-2, a secretory protein and low density lipoprotein receptor related protein-2 (LRP-2), an endocytic receptor, were immunolocalized at the light microscopic level within epithelial cells of the cauda epididymidis and vas deferens. Principal cells showed numerous intensely reactive lysosomes for cathepsins A, D, and SGP-1 in all regions of the cauda and vas deferens, and for cathepsin B only in the cauda epididymidis. Basal cells were intensely reactive for cathepsin A, unreactive for cathepsins D and B, and weakly reactive for SGP-1 in the cauda region. In the vas deferens, these cells were intensely reactive for cathepsin A and SGP-1 and unreactive for cathepsin B; in the case of cathepsin D basal cells were weakly reactive in the proximal vas deferens, but intensely reactive in the middle and distal vas deferens. Clear cells, present in the cauda region and proximal vas deferens, were intensely reactive for cathepsin A, weakly reactive for SGP-1 and unreactive for cathepsins D and B, while narrow cells found mainly in the proximal vas deferens were intensely reactive for cathepsins A, D and SGP-1 and unreactive for cathepsin B. Thus, the expression of different lysosomal enzymes in the cauda epididymidis and vas deferens is not only cell but also region specific suggesting differences in the type of substrates internalized by these cells. SGP-2, a secretory protein, showed a checkerboard-like staining pattern in the cytoplasm of principal cells of the cauda epididymidis, while the cytoplasm of all principal cells were intensely reactive in the vas deferens. This type of reaction as well as staining of sperm suggest that SGP-2 is secreted into the lumen where it functions in relation to sperm. The endocytic receptor LRP-2 was noted only on the apical surface of principal cells of the cauda and vas deferens and in spherical structures indicative of endosomes suggestive of their role in the uptake of various ligands, including SGP-2, for which it has a high binding affinity. Thus SGP-2 in the cauda and vas deferens is not only secreted but endocytosed by principal cells, suggestive of an active turnover in the lumen. In summary, the epithelial cells of the cauda and vas deferens show marked differences in

expression of lysosomal proteins, SGP-2 and LRP-2 suggestive of differences in their functional activity while sperm are stored and protected in these regions.

4.2. INTRODUCTION:

The cauda epididymidis and vas deferens are major storage sites for sperm in many species, including humans (Turner, 1995). The epithelial cells lining these regions must, therefore, play important roles in providing sperm with a suitable environment for their survival and protection. Principal cells, the major cell type of the cauda and vas deferens have been shown to be active in synthesis of proteins destined to be secreted into the lumen via a merocrine manner (Kennedy and Heidger, 1979; Wenstrom and Hamilton, 1984; Robaire and Hermo, 1988). Apocrine secretion has also been suggested for principal cells of the vas deferens, whereby cytoplasmic blebs of the apical surface detach from principal cells to be liberated into the lumen (Agrawal and Vanha-Pertulla, 1988). In the mouse, a protein without a signal peptide sequence and glycosylation site limiting it to the cytosol, called mouse vas deferens protein (MVDP), has been postulated to be secreted via this mechanism (Manin et al., 1995). However, little is known about other specific proteins secreted by principal cells.

The lumen of the cauda epididymidis and vas deferens is not only modified by the secretion of proteins therein, but also by the endocytosis of substances via coated pits on the apical surface of principal cells and their subsequent appearance in the endocytic apparatus composed of endosomes, multivesicular bodies (MVBs) and lysosomes where they are presumably degraded (Friend and Farquhar, 1967; Friend, 1969; Moore and Bedford, 1979; Hermo and DeMelo, 1987). While lysosomes are abundant in principal cells (Hamilton, 1975), and studies have documented a cell and region specific distribution of lysosomal integral membrane proteins (Suarez-Quian et al, 1992), the nature of the soluble lysosomal proteins has not been analyzed, nor have receptors on the apical cell surface of principal cells for receptor-mediated endocytosis been described.

Principal cells of the vas deferens have been shown to have region specific differences in their structural features (Flickinger, 1973; Hamilton, 1975; Kennedy and Heidger, 1979; Robaire and Hermo, 1988). Those of the proximal vas deferens, resemble principal cells of the cauda epididymidis with an abundance of basal rough endoplasmic reticulum (rER), a large supranuclear Golgi apparatus and elaborate endocytic apparatus. However, in the middle vas deferens, these cells, in addition, contain an abundance of smooth endoplasmic reticulum (sER) which appears to be involved in steroid synthesis

(Hamilton et al., 1969; Hamilton and Fawcett, 1970; Andonian and Hermo, 1999a). In the distal vas deferens, large dilated intercellular spaces are evident between adjacent principal cells, as well as aquaporin water channels, suggesting the transport of water from the lumen to the subepithelial lamina propria (Brown et al, 1993; Andonian and Hermo, 1999a). The epithelium of the vas deferens also contains basal cells throughout its entire length, while narrow and clear cells are present in the proximal vas deferens (Flickinger, 1973; Hamilton, 1975; Kennedy and Heidger, 1979; Robaire and Hermo, 1988; Andonian and Hermo, 1999a). Recently, it has been demonstrated that glutathione S-transferases, possibly serving to protect sperm, are expressed in a cell and region specific manner in the different regions of the vas deferens as well as cauda epididymidis (Hales et al, 1980; Andonian and Hermo, 1999b). All of these cell types have their equivalent in humans but little is known about their respective functions in the different species (Popovic et al., 1973; Hoffer, 1976).

In the epididymis, region specific differences for principal, clear and basal cells have been noted for synthesis of secretory proteins, lysosomal proteins as well as cell surface receptors suggesting that sperm encounter a continuously changing environment as they traverse the duct *en route* to maturation (Hermo et al., 1991, 1992a,b; Igdoura et al., 1995; Morales et al., 1996). However, this type of study has not been performed in the different regions of the cauda or vas deferens.

The purpose of the present study was to examine the expression of secretory and lysosomal proteins, and cell surface receptors in the various epithelial cell types of the cauda epididymidis and vas deferens, areas where sperm are stored. Use of Bouin-fixed paraffin-embedded tissue in conjunction with light microscope immunocytochemistry revealed major differences in expression of various lysosomal and secretory proteins and endocytic receptors often in a cell and region specific manner.

4.3. MATERIALS AND METHODS

4.3.1. Tissue Preparation for Light Microscopy:

Four adult male Sprague Dawley rats (350-450 g) obtained from Charles River Laboratories (St. Constant, QC) were anesthetized with an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON). The cauda epididymidis and vas deferens were fixed by perfusion with Bouin's fixative for 10 minutes through the abdominal aorta in an anterograde manner. After perfusion, the cauda and vas deferens of both sides of each animal were removed and immersed in Bouin's fixative for another 24 hours. Prior to immersion, the vas deferens was divided arbitrarily into proximal, middle, and distal regions so that the entire duct of each animal would fit into one paraffin block. In addition, the cauda epididymidis was cut in thirds along its long axis in such a way that the proximal, middle, and distal regions would be included for examination. After fixation, the tissues were dehydrated and eventually embedded in paraffin. The vas deferens was embedded to allow sectioning along its long axis. To better visualize the histology of the epithelial cells of the cauda and vas deferens, sections of these tissues were stained with the Periodic Acid Schiff technique (PAS) as outlined by Leblond (1950).

4.3.2. Immunoperoxidase procedure:

Paraffin sections, 5 μm thick, were deparaffinized in xylene and hydrated in a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished in 70% ethanol containing 1% (v/v) H_2O_2. Once hydrated, the tissue sections were incubated in a 300 mM glycine solution to block free aldehyde groups.

Immunoperoxidase staining of the cauda epididymidis and vas deferens was carried out according to the procedure of Oko and Clermont (1989). Polyclonal antibodies reactive against cathepsins A, D, and B, SGP-1 and SGP-2 and LRP-2 were used in these experiments. The anti-human cathepsin D antibody was obtained from Calbiochem (La Jolla, CA) and has been localized in lysosomes (Igdoura et al., 1995). The rabbit anti-rat procathepsin B antibody was generously provided by Dr. John Mort (Shriners Hospital, Montreal, Canada) and its purification and specificity have been characterized (Rowan et al., 1992). Anti-SGP-1 and SGP-2 antibodies were kindly provided by Dr. M.D. Griswold (Washington State University, Pullman, WA). Anti-SGP-1 and anti-SGP-2 antibodies have

been well characterized in Sylvester et al. (1989) and Sylvester et al. (1984, 1991), respectively. Anti-LRP-2 antibody was kindly provided by Dr. Scott Argraves (Medical University of South Carolina, Charleston, SC) (Kounnas et al., 1995; Morales et al., 1996). Anti-cathepsin A antibody was generously provided by Dr. Y. Suzuki (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and its specificity is discussed in Satake et al. (1994).

Before immunostaining, tissue sections were blocked for 15 min with 10% goat serum in Tris-HCl-buffered saline (TBS). This and subsequent treatments were accomplished by placing 100 µl of a solution onto a coverslip and overturning the tissue face of the slide onto the drop, thus ensuring that the entire tissue was treated with minimal fluid (Oko and Clermont, 1989). Coverslips were removed by dipping the slides in TBS containing 1% Tween-20 (TWBS). Sections were then incubated in a 37°C humidified incubation chamber for 1.5 hr with their respective polyclonal antibodies (diluted 1/100 in TBS for cathepsin A, B, LRP-2, and 1/50 in TBS for cathepsin D, SGP-1, SGP-2). After four 5 min washes in TWBS, sections were once again blocked with 10% goat serum in TBS. They were then incubated for 30 min (at 37°C) with goat anti-rabbit IgG conjugated to peroxidase (Sigma Chemical Co., St. Louis, MO) diluted 1:250 in TBS. This was followed by four 5 min washes in TWBS.

The final reaction product was achieved by incubating the sections for 10 min in 500ml of TBS containing 0.03% H_2O_2, 0.1M imidazole, and 0.05% diaminobenzidine tetrahydrochloride (Sigma), pH 7.4. Slides were then washed in distilled H_2O and counterstained with 0.1% methylene blue for 1 min. The tissue was dehydrated by passing slides through a graded ethanol series, after which the sections were immersed in xylene and mounted with Permount. Tissue sections incubated without the primary antibody were used as controls.

4.4. RESULTS:

4.4.1. Structural appearance of the epithelium of the cauda epididymidis and vas deferens in PAS-stained material:

In PAS-stained tissue, the structural features and organization of the different epithelial cell types were well visualized in the different regions of the cauda epididymidis and vas deferens. Principal cells showed a gradual increase in height from cuboidal in the middle cauda region (Fig. 1a) to tall columnar in the distal vas deferens (Fig. 1f). Their microvilli also increased in height, and their nucleus shifted from a basal location to a mid-position in the cell (compare Figs. 1a-f). In the middle region of the vas deferens, the cell surface of principal cells showed apical blebs or protrusions of cytoplasm, some of which appeared to be detached from the cell (Fig. 1d). In the middle and distal vas deferens, dilated intercellular spaces were noted between principal cells (Figs. 1 d-f). Clear cells, intensely stained with PAS due to abundant lysosomes rich in glycoproteins, were abundant in the cauda region (Fig. 1a), became sparse in the proximal vas deferens, and were absent in other regions. Narrow cells, few in number, were mainly found in the proximal vas deferens (Fig. 1b). Basal cells were present in all regions (Figs. 1a-f). The lamina propria, well stained with PAS due to abundance of glycoproteins, consisted of several concentric cellular and acellular layers, subjacent to which in the vas deferens were pale stained smooth muscle cell layers (Figs. 1a-f). Large vascular channels were noted in the lamina propria of the distal vas deferens (Fig. 1e).

4.4.2. Immunocytochemical localization of lysosomal proteins:

With anti-cathepsin A antibody, principal cells of the entire cauda epididymidis and all regions of the vas deferens showed numerous intensely reactive lysosomes (Figs. 2a-c). In the case of basal, clear and narrow cells, intense reactivity was noted over these cells (Figs. 2a-c). With anti-cathepsin D antibody, principal cells also contained numerous intensely reactive lysosomes in all regions of the cauda and vas deferens (Figs. 3a-c). However, while clear cells were unreactive (Figs. 3 a,b), narrow cells were intensely reactive (not shown). Basal cells were unreactive in the cauda epididymidis, weakly reactive in proximal vas deferens, and intensely reactive in the middle and distal vas deferens (Figs. 3a-c).

Principal cells of the cauda epididymidis contained numerous intensely reactive

lysosomes for anti-cathepsin B antibody (Fig. 4a), however, in the proximal vas deferens, these cells showed few intensely reactive lysosomes (not shown), while in the middle and distal vas deferens intensely reactive lysosomes were not present (Figs. 4b, c). Clear, narrow (not shown) and basal cells were unreactive in all regions (Figs. 4a-c).

Numerous intensely reactive lysosomes were noted in principal cells of all regions of the cauda and vas deferens with anti-SGP-1 antibody, and while clear cells were weakly reactive, narrow cells were intensely reactive (Figs. 5a-c). Basal cells were weakly reactive in the cauda epididymidis, but intensely reactive in the vas deferens (Figs. 5a-c).

4.4.3. Immunocytochemical localization of SGP-2 and LRP-2:

Staining for anti-SGP-2 antibody revealed that all principal cells were uniformly and intensely reactive in all regions of the vas deferens (Figs. 6a, b), while a checkerboard staining pattern was seen in all regions of the cauda epididymidis where principal cells were intensely, moderately, or weakly reactive (not shown). Clear, narrow and basal cells were unreactive (Figs. 6a, b). With anti-LRP-2 antibody, an intense reaction was noted over principal cells of all regions of the cauda epididymidis and vas deferens in the form of a dense apical band, as well as small spherical apically located bodies identified as endosomes (Figs. 7a, b). Clear, narrow and basal cells were unreactive (Figs. 7a, b).

4.5. FIGURES, TABLES, AND LEGENDS:

Fig. 1: Light micrographs of the middle cauda epididymidis **(a)**, proximal vas deferens **(b)**, proximal **(c)** and distal **(d)** halves of the middle vas deferens, and proximal **(e)** and distal **(f)** halves of the distal vas deferens stained with the PAS technique. Principal cells (P) appear in all regions and show a progressive increase in height from **(a)** to **(f)**. Their nuclei (n) change from a basal location **(a-c)** to a gradual mid-position in the cell **(d-f)**. Their microvilli (mv) also show a progressive increase in size from **(a)** to **(f)**, but in **(d)** numerous blebs (small curved arrows) extend from the apical surface of principal cells, some of which appear to be free in the lumen (Lu). Principal cells **(d-f)** show empty looking dilated intercellular spaces (stars) from the mid to basal position of the epithelium. Clear cells (C), showing intense staining with PAS technique, are present in **(a)**, while narrow cells (large curved arrows) are seen in **(b)** and **(c)**. Basal cells (arrowheads) are noted in all regions. The lamina propria (Lp), immediately subjacent to the epithelium, is more intensely stained than the smooth muscle cell layers (Sm). Note a large vascular channel (va) in **(e)**. IT, intertubular space. X544 for each.


Lu
C
IT
P
1a
mv
Sm
b
Lu
mv
c
Sm
Lu
P
P
d
mv
va
e
Sm
mv
P
f

Fig. 2: Distal cauda epididymidis **(a)**, and proximal **(b)** and distal **(c)** vas deferens immunostained with anti-cathepsin A antibody. In all regions, principal cells (P) show numerous intensely reactive lysosomes (small arrows), while clear (large arrows), narrow (curved arrow), and basal (arrowheads) cells are intensely reactive. Note large dilated intercellular spaces in the epithelium of the distal vas deferens (stars). Asterisks, sperm in lumen; Lp, lamina propria; Sm, smooth muscle cell layers. X 544 for each.

Fig. 3: Middle cauda epididymidis **(a)** and proximal **(b)** and distal **(c)** vas deferens immunostained with anti-cathepsin D antibody. Principal cells (P) show numerous intensely reactive lysosomes (small arrows) in all regions, while basal cells (arrowheads) are unreactive in **(a)**, weakly reactive in **(b)**, and intensely reactive in **(c)**. Clear cells (large arrows) are unreactive. Note dilated intercellular spaces in the distal vas deferens (stars). Asterisks, sperm in lumen; IT, intertubular space; Lp, lamina propria; Sm, smooth muscle cell layers; va, vascular channel. X 544 for each.

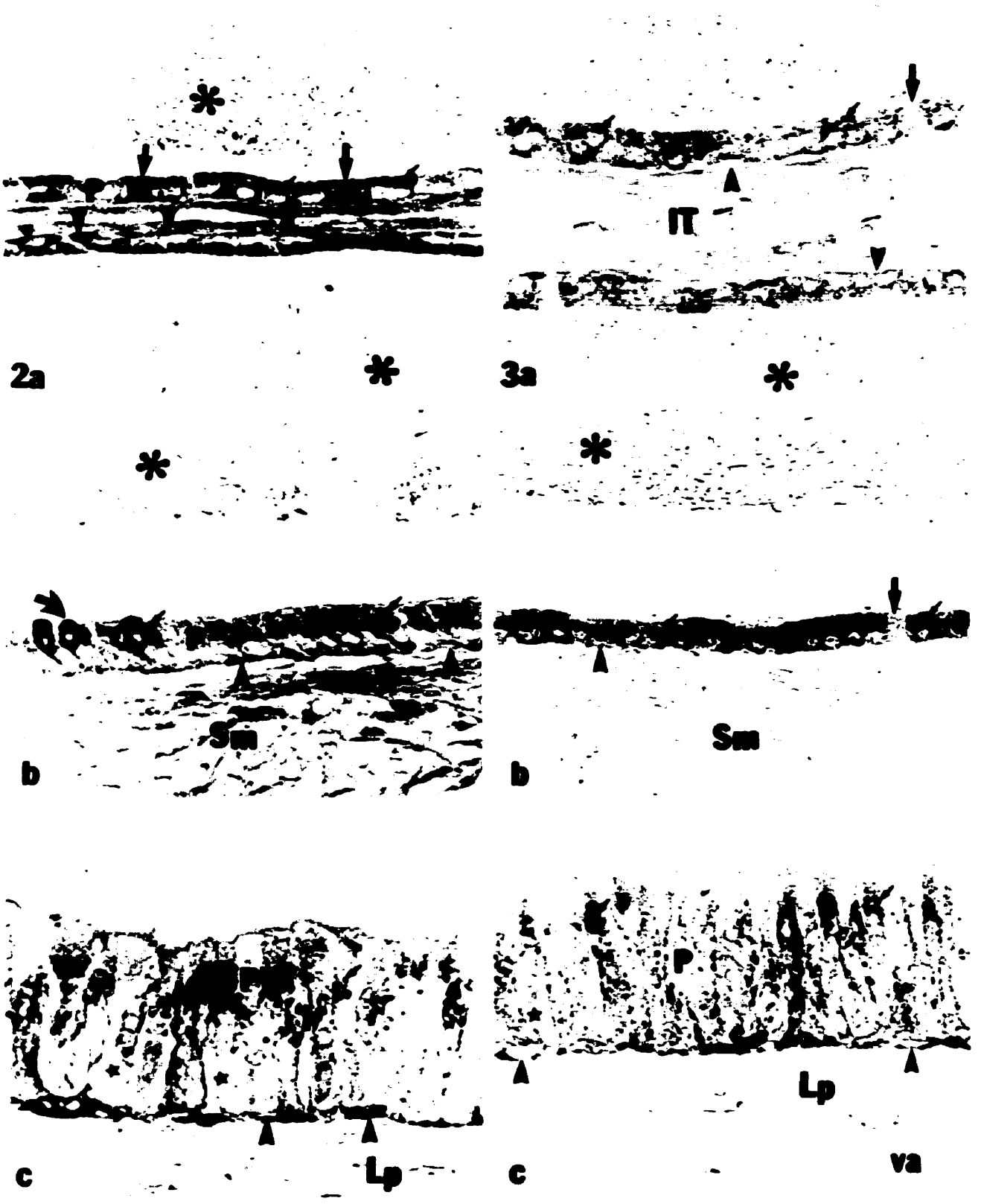
2a
3a
IT
b
Sm
b
Sm
c
Lp
c
P
Lp
va

Fig. 4: Distal cauda epididymidis **(a)** and middle **(b)** and distal **(c)** vas deferens immunostained with anti-cathepsin B antibody. Principal cells (p) in **(a)** show numerous intensely reactive lysosomes (small arrows), while reactive lysosomes are not apparent in **(b)** and **(c)**. Basal cells (arrowheads) appear unreactive in **(a-c).** Clear cells (large arrows) are unreactive in **(a)**. Note apical blebs (curved arrows) on surface of principal cells and dilated intercellular spaces (stars) between principal cells in **(b)** and **(c)**. Asterisks, sperm in lumen; IT, intertubular space; Lp, lamina propria; n, nucleus of principal cells; Sm, smooth muscle cell layers. X 544 for each.

Fig. 5: Distal cauda epididymidis **(a)** and proximal **(b)** and distal **(c)** vas deferens immunostained with anti-SGP-1 antibody. Principal cells (p) exhibit numerous intensely reactive lysosomes (small arrows) in all regions. Basal cells (arrowheads) are weakly reactive in **(a),** and intensely reactive in **(b)** and **(c)**. Clear cells (large arrows) are weakly reactive in **(a)**. Note dilated intercellular spaces (stars) in **(c)**. Asterisks, sperm in lumen; Lp, lamina propria; Sm, smooth muscle cell layers; va, vascular channel. X 544 for each.

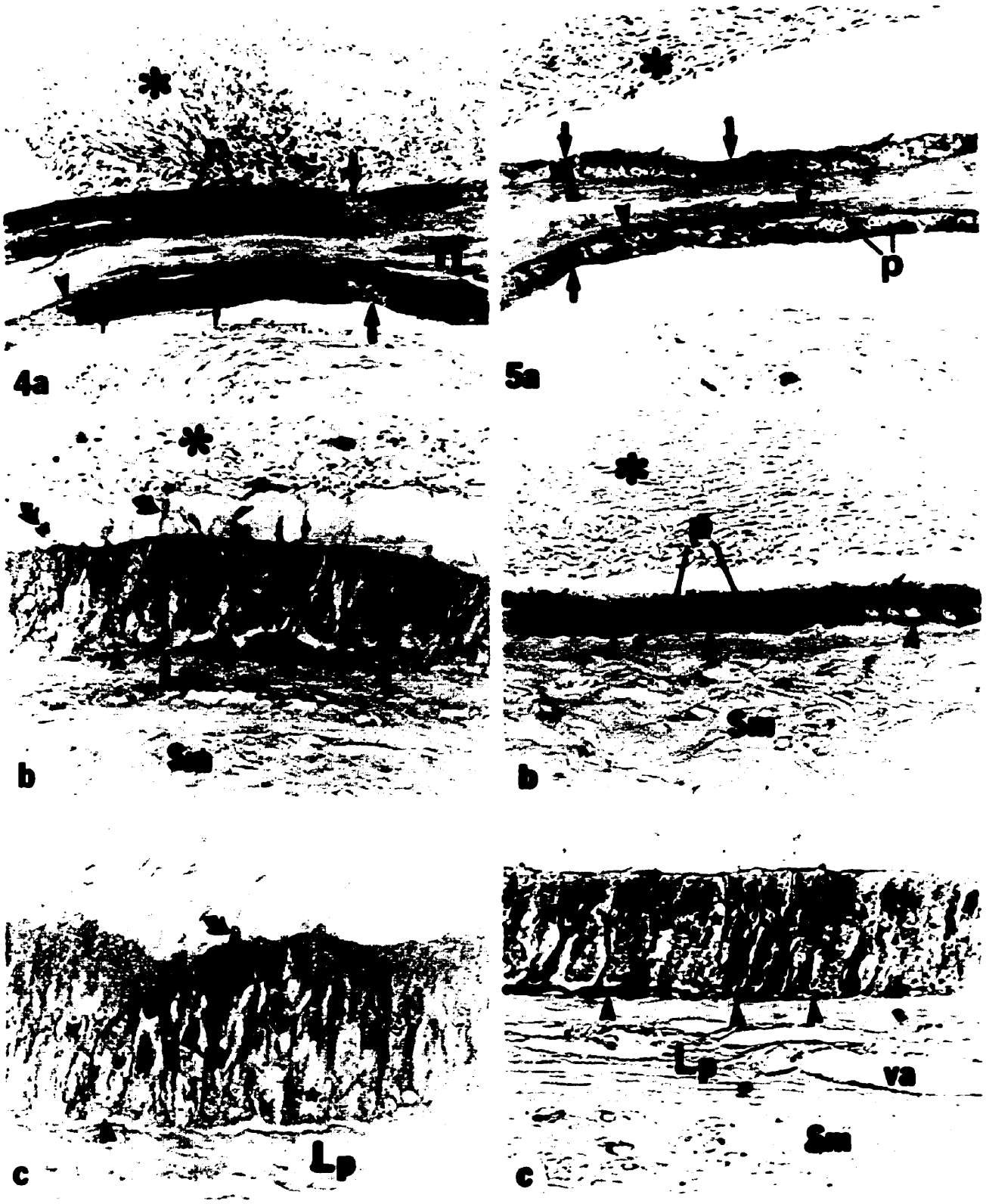
4a
5a
p
b
Sm
b
Sm
c
Lp
c
Lp
va
Sm

Fig. 6: Proximal **(a)** and distal **(b)** regions of the vas deferens immunostained with anti-SGP-2 antibody. All principal cells (p) show intense reaction throughout their cytoplasm, while basal cells (arrowheads) are unreactive. Reaction appears over spermatozoa in the lumen (asterisks) and smooth muscle cell layers (Sm). Lp, lamina propria; Sm, smooth muscle cell layers; star, dilated intercellular space. X 544 for each.

Fig. 7: Middle cauda epididymidis **(a)** and distal vas deferens **(b)** immunostained with anti-LRP-2 antibody. In **(a)**, principal cells (p) present an intense apical band of reaction product (open arrow) as well as small spherical reactive bodies identified as endosomes (small arrows), while clear (large arrows) and basal (arrowheads) cells are unreactive. In **(b)**, principal cells (p) show an intense apical band of reaction product (open arrow) as well as small spherical reactive endosomes (small arrows). Basal cells (arrowheads) are unreactive. Asterisks, sperm in lumen; IT, intertubular space; Lp, lamina propria; stars, dilated intercellular spaces. X 544 for each.

P
6a
Lp
P
b
P
Lp
P
P
7a
b

Fig. 8: Diagrammatic representation of the staining pattern for **(a)** lysosomal proteins and **(b)** SGP-2 and LRP-2 of the cauda epididymidis and proximal (PVD), middle (MVD), and distal (DVD) vas deferens. Principal cells are represented with microvilli and show a progressive increase in size from cauda to DVD. Clear cells, present only in the cauda and PVD are represented without microvilli. Narrow cells, found only in PVD, are represented as goblet shaped cells without microvilli and are situated at the right edge of the PVD epithelium. Basal cells, found throughout, are represented as hemispherical cells. In **(a)**, reactive lysosomes within principal cells are represented as dark dots, with four being numerous, two few in number and none representing absence of reactive lysosomes. While in the case of basal, narrow and clear cells, the entire cell cytoplasm was often stained. The latter observation is due to the fact that these cells contain many lysosomes resulting in a uniform reaction that often appears over the entire cytoplasm, often preventing visualization of individual lysosomes, while for clear, narrow and basal cells, it refers to the intensity of reactivity over the cell cytoplasm. With cathepsin A (**Cath A**), principal cells throughout the cauda and vas deferens show numerous intensely reactive lysosomes, while clear, narrow and basal cells are intensely reactive in all regions. With cathepsin D (**Cath D**), principal cells reveal numerous intensely reactive lysosomes in all regions of the cauda and vas deferens, and while clear cells are unreactive, narrow cells are intensely reactive. Basal cells are unreactive in the cauda region but become intensely reactive in the MVD and DVD. With cathepsin B (**Cath B**), principal cells show numerous intensely reactive lysosomes in the cauda region, few in the PVD and none in the MVD and DVD. Clear, narrow and basal cells are unreactive. With sulfated glycoprotein-1 (**SGP-1**), principal cells present numerous intensely reactive lysosomes in the cauda and vas deferens, and while clear cells are weakly reactive narrow cells are intensely reactive. Basal cells are weakly reactive in the cauda but intensely reactive in the vas deferens.

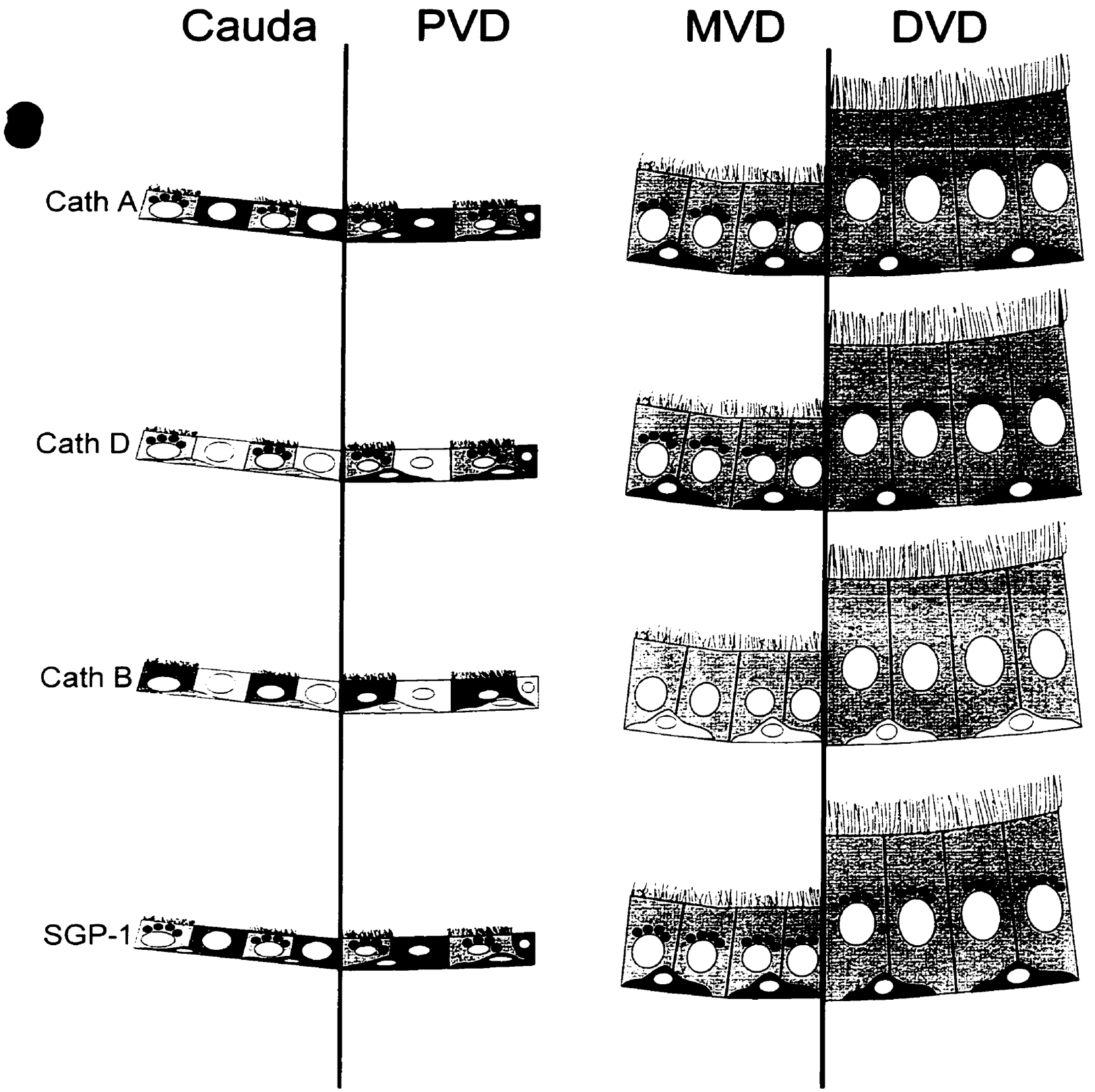
Cauda
PVD
MVD
DVD
Cath A
Cath D
Cath B
SGP-1

Fig. 8 b: Principal cells reveal a checkerboard-like staining pattern for sulfated glycoprotein-2 (**SGP-2**) in the cauda, while all principal cells are intensely reactive in the vas deferens. Clear, narrow and basal cells are unreactive. With low density lipoprotein receptor related protein-2 (**LRP-2**), principal cells show an intense staining of numerous endosomes represented as small dark dots in the apical region of the cell. Clear, narrow and basal cells are unreactive.

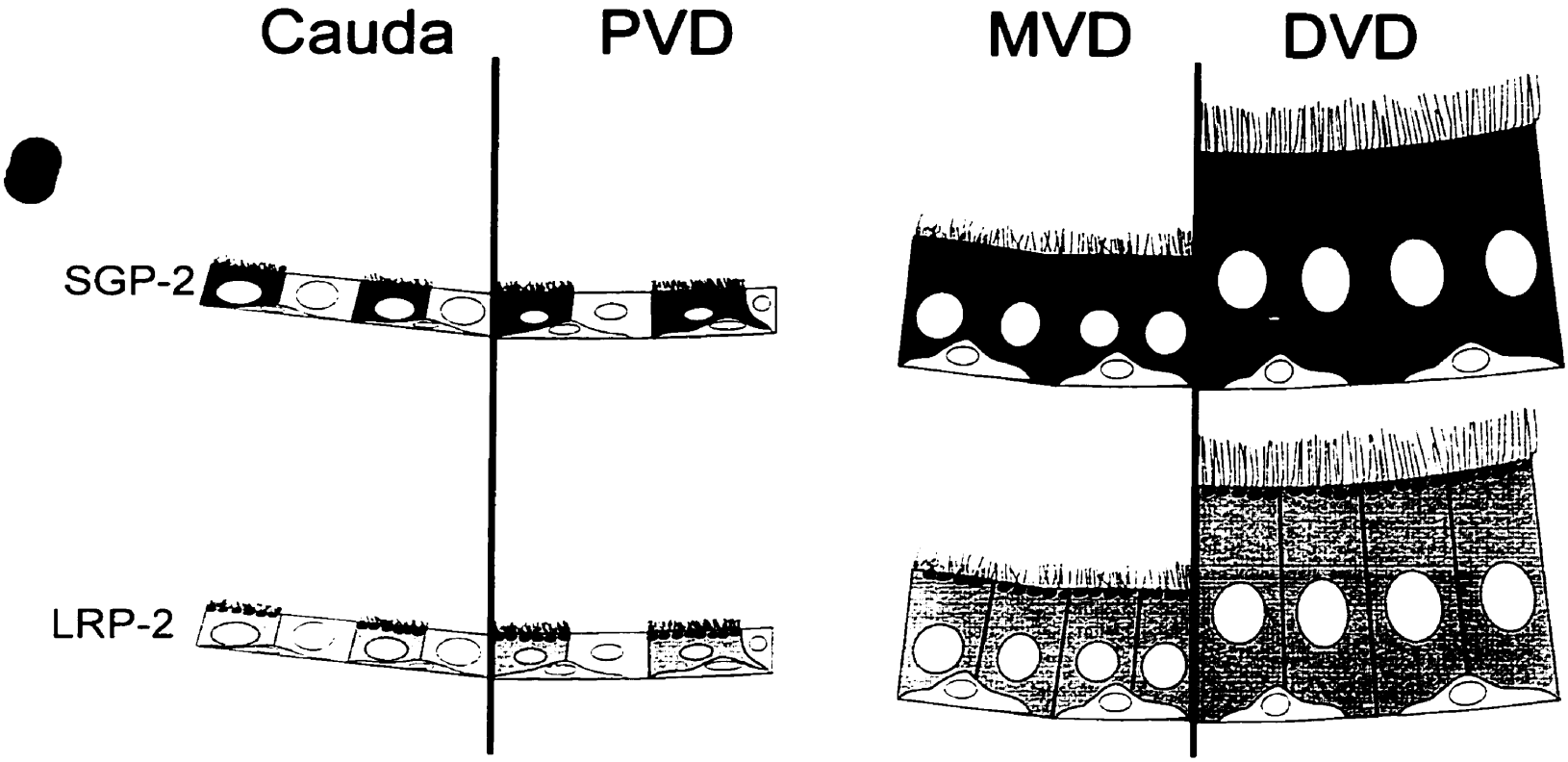
Cauda
PVD
MVD
DVD
SGP-2
LRP-2

TABLE 1: Differential expression of various lysosomal proteins in epithelial cells of the cauda epididymidis and various regions of the vas deferens.

Regions	Ab / Cell Types	Cath A	Cath D	Cath B	SGP-1
Cauda[1]	Principal	++[2]	++	++	++
	Basal	++	-	-	+
	Clear	++	-	-	+
PVD	Principal	++	++	+	++
	Basal	++	+	-	++
	Clear	++	-	-	+
	Narrow	++	++	-	++
MVD/DVD	Principal	++	++	-	++
	Basal	++	++	-	++

[1] Cauda, PVD, MVD, and DVD correspond to all regions of the cauda epididymidis, and proximal, middle and distal vas deferens regions, respectively.

[2] In the case of principal cells, the number of plus signs is directly proportional to the relative number of reactive lysosomes with (++) being high, (+) low, and (-) absent, while for basal, clear, and narrow cells, it refers to the intensity of reactivity over the cell cytoplasm.

TABLE 2: Differential expression of SGP-2 and LRP-2 in epithelial cells of the cauda epididymidis and various regions of the vas deferens.

Regions	Ab / Cell Types	SGP-2	LRP-2
Cauda[1]	Principal	(CB)[2]	++(A)[3]
	Basal	-	-
	Clear	-	-
PVD	Principal	++[4]	++(A)
	Basal	-	-
	Clear	-	-
	Narrow	-	-
MVD/DVD	Principal	++	++(A)
	Basal	-	-

[1] Cauda, PVD, MVD, and DVD correspond to all regions of the cauda epididymidis, and proximal, middle, and distal vas deferens regions, respectively.

[2] (CB) indicates a checkerboard staining pattern.

[3] (A) indicates an apical reaction.

[4] The plus signs (++) indicates an intense reaction over principal cells, while the minus sign (-) indicates the absence of reaction.

4.6. DISCUSSION:

In the present study with PAS staining, it was readily observed that several structural changes take place in the epithelium from the proximal cauda epididymidis to the distal vas deferens including size of principal cells and their microvilli and shift of their nucleus from a basal to mid position in the cell. Basal cells, present in all areas, become more closely aligned in the middle and distal vas deferens, where, in addition, apical blebs of principal cells are especially prominent. Clear cells are abundant in the cauda epididymidis, but are few in number along with narrow cells in proximal vas deferens. These observations along with similar observations of others (Hamilton, 1975; Hamilton and Cooper, 1978; Kennedy and Heidger, 1979; Robaire and Hermo, 1988; Andonian and Hermo, 1999a) will serve as a framework for understanding the subsequent sections of the discussion dealing with the light microscope immunocytochemistry, where preservation of the tissue is not optimal.

4.6.1. Distribution of lysosomal proteins:

Principal cells of the cauda epididymidis and vas deferens have numerous lysosomes in their supranuclear cytoplasm as revealed by electron microscopic studies (Friend and Farquhar, 1967; Hamilton, 1975; Hermo and De Melo, 1987; Robaire and Hermo, 1988). These cells are also equipped with an endocytic apparatus whereby tracers introduced into the lumen of the duct appear temporally and sequentially within coated pits and vesicles, endosomes, MVBs, and eventually within lysosomes (Friend and Farquhar, 1967; Friend, 1969; Moore and Bedford, 1979; Hermo and DeMelo, 1987). Clear cells also perform this function but to a greater degree than principal cells (Moore and Bedford, 1979). Clear cells endocytose immobilin in the distal cauda epididymidis and in this and other caudal regions take up the contents of cytoplasmic droplets released from sperm in the lumen as well as other specific proteins (Hermo et al., 1988, 1992b; Vierula et al., 1995). Narrow and basal cells also show coated pits, endosomes, MVBs and lysosomes and thus appear to be capable of endocytosis (Robaire and Hermo, 1988; Andonian and Hermo, 1999a). In these cell types as well as others, lysosomal enzymes are derived from the Golgi apparatus and ferried via small vesicles from the trans Golgi network, often bound to mannose-6- phosphate receptors, to multivesicular bodies and hence eventually appear within lysosomes (Griffiths and Simons, 1986; Hermo et al., 1992a; Farquhar and Hauri, 1997). However, despite the presence of lysosomes in epithelial cells of the epididymis and vas deferens, the localization

of soluble lysosomal proteins has yet to be performed.

In the present study, localization was performed with the light microscope, however, anti-cathepsin D, B and SGP-1 antibodies have been examined at the electron microscopic level in the epididymis where they were localized within lysosomes of the epithelial cells and not endosomes (Hermo et al., 1992a; Igdoura et al., 1995). In the case of principal cells, reactivity was in the form of small spherical bodies corresponding to lysosomes, while in the case of basal, narrow and clear cells, the entire cell cytoplasm was often stained. The latter observation is due to the fact that these cells contain many lysosomes (Flickinger, 1973; Hamilton, 1975; Kennedy and Heidger, 1979; Hermo et al., 1988; Robaire and Hermo, 1988) resulting in a uniform reaction that often appears over the entire cytoplasm, and thus preventing visualization of individual lysosomes. Although the present data are qualitative in nature and not quantitative, the reaction was assigned subjective values and reflects the relative abundance of these proteins in the different cell types and regions (Table 1). In addition, while the data indicate the distribution of these enzymes in the different cell types and regions, it does not address the enzyme activity of each. Such studies on a cell type basis have not been performed and are beyond the scope of this work.

In the lysosomes of mammalian cells, proteolytic enzymes named cathepsins play a role in the intracellular degradation of exogenous and endogenous proteins (Kirschke et al., 1980; Kominami et al., 1991) and activation of enzyme precursors (Barrett and Kirschke, 1981). They are also involved in metastasis of cancer cells (Domagala et al., 1992; Rochefort, 1994). There is also evidence that cathepsins play a limited role in antigen processing and inflammation of tissue, contributing to the overall immune response (Takahashi et al., 1989).

Cathepsin A, also known as protective protein, is a lysosomal glycoprotein forming a high molecular weight complex with lysosomal β-galactosidase and neuraminidase (Satake et al., 1994). In this complex cathepsin A functions as a protective protein to regulate the expression of these enzymes in lysosomes by stabilizing β-galactosidase and activating neuraminidase (Galjart et al., 1991; Itoh et al., 1995), in addition to catalytic activities for the hydrolysis of peptide and ester bonds such as those of acid carboxypeptidase, neutral esterase and carboxyl-terminal deamidase (Galjart et al., 1991; Itoh et al., 1995). It may also serve as an extra-lysosomal regulatory protein (Satake et al., 1994). Mutations or absence of

cathepsin A results in deficiency of these two lysosomal enzymes and in a disorder termed galactosialidosis (d'Azzo et al., 1995; van der Spoel et al., 1998). In the present study, numerous intensely reactive lysosomes were noted in principal, clear, basal and narrow cells of the cauda and vas deferens for cathepsin A (Table 1, Fig. 8a), indicating a universal distribution and important role for cathepsin A in these cell types. It may thus be suggested that substances endocytosed by these cells require the presence of cathepsin A in their lysosomes.

Cathepsin D, an aspartyl endopeptidase with a molecular weight of 42KD and optimal activity at pH 3.8, has been studied in many tissues where it has been localized in lysosomes (Srivastava and Ninjoor, 1982; Kominami et al., 1991). In the present study, principal cells in all regions examined showed numerous intensely reactive lysosomes for anti-cathepsin D antibody (Table 1, Fig. 8a). This finding was in contrast to that observed in the rest of the epididymis, where cathepsin D showed a progressive increase in reactivity in principal cells from the initial segment to the proximal cauda epididymidis (Igdoura et al., 1995). In the present study, basal cells showed a progressive increase in reactivity from the cauda to the distal vas deferens (Table 1, Fig. 8a). In the rest of the epididymis, these cells also showed region specific differences with little or no reactivity in the initial segment, corpus and proximal cauda, but intense reactivity in the intermediate zone and proximal caput regions (Igdoura et al., 1995). Clear cells of the cauda and vas deferens regions were unreactive (Table 1, Fig. 8a), unlike the case in the caput epididymidis where they were intensely reactive, but similar to the corpus epididymidis where they were unreactive (Igdoura et al., 1995). Taken together, these data reveal cell and region specific differences in the expression of cathepsin D in the cauda and vas deferens and suggest an important role for this enzyme in lysosomes of principal and basal cells, but not clear cells. The data also suggest substrate specificity with respect to the type of substances taken up by the different cell types of these regions.

Cathepsin B, a cysteine proteinase with optimal activity at pH 5.5-6.5, is present in lysosomes of many cell types (Scott et al., 1987; Yokota and Kato, 1988; Kominami et al., 1991). In the present study, there was a progressive decrease in reactive lysosomes in principal cells from the cauda region to the distal vas deferens (Table 1, Fig. 8a). This finding suggests that cathepsin B plays a more important role in the cauda region than the vas

deferens. In comparison, principal cells showed numerous intensely reactive lysosomes for cathepsin B in the initial segment and caput regions, but none in the corpus epididymidis (Igdoura et al., 1995). Basal and clear cells were unreactive in the cauda epididymidis and vas deferens (Table 1, Fig. 8a), and this was also noted in the rest of the epididymis (Idgoura et al., 1995). These data thus suggest cell and region specificity with respect to the localization of cathepsin B and indicate substrate specificity with respect to the substances endocytosed by the epithelial cells of the cauda and vas deferens. It has been suggested that epithelial cells of the epididymis secrete pro-forms of cathepsin B into the epididymal lumen (Tomomasa et al., 1994). In the present study, using an anti- procathepsin B antibody, we noted diffuse cytoplasmic staining of principal cells of the cauda which may be suggestive of secretion of cathepsin B into the lumen. However, electron microscopic confirmation of this observation would be required.

Sulfated glycoprotein-1 (SGP-1) is a heavily glycosylated and sulfated protein (Sylvester et al., 1989). It has four domains that share substantial sequence similarity with the precursor of human sphingolipid activator proteins called prosaposin (Morimoto et al., 1988). Present in lysosomes, prosaposin is proteolytically cleaved into four smaller proteins called saposins A, B, C, and D, each of which are believed to solubilize certain lipids so that they may be acted upon by specific lysosomal hydrolases (O'Brien and Kishimoto, 1991). Absence of saposins leads to various clinical conditions such as Gaucher disease and metachromatic leukodystrophy (Stevens et al., 1981; Christomanou et al., 1986). In the present study, numerous reactive lysosomes were noted in principal cells of the cauda and vas deferens regions (Table 1, Fig. 8a). This was also the case noted for SGP-1 in principal cells along the entire epithelial duct (Hermo et al., 1992a), suggesting an important role for SGP-1 in the processing of substances internalized by these cells. Clear cells were weakly reactive in the cauda and proximal vas deferens (Table 1, Fig. 8a), however, intense reactivity was noted for these cells in the caput and corpus epididymal regions (Hermo et al., 1992a). While basal cells were weakly reactive in the cauda region, they showed intense reactivity in the vas deferens (Table 1, Fig. 8a). In the epididymis, basal cells were intensely reactive in most epididymal regions (Hermo et al., 1992). Thus expression of SGP-1 in the case of clear and basal cells in the cauda and vas deferens appears to be region specific suggesting substrate specificity with respect to the substances endocytosed by these cells, as

also evidenced for cathepsins D and B.

In the present study, narrow cells were intensely reactive for cathepsin A, D, and SGP-1, while unreactive for cathepsin B (Table 1, Fig. 8a). Cells with a similar appearance and features, such as numerous small cup shaped vesicles in their apical cytoplasm, have been noted in the initial segment and intermediate zone of the epididymis. However, while the latter expressed SGP-1 and cathepsin D, they did not express cathepsin B (Adamali and Hermo, 1996), suggesting different functions performed by narrow cells of these different regions. In common, however, is the finding that narrow cells of the vas deferens express H+ ATPase suggestive of a role in acidification of the lumen (Breton et al., 1996) and this also appears to be the case for narrow cells of the initial segment and intermediate zone which express carbonic anhydrase II (Adamali and Hermo, 1996).

4.6.2. Distribution of SGP-2 and LRP-2:

Sulfated glycoprotein-2 (SGP-2), also designated as clusterin or apolipoprotein J, has been shown to be synthesized by epididymal principal cells (Sylvester et al., 1984, 1991; Tung and Fritz, 1985; Mattmueller and Hinton, 1991; Hermo et al., 1991; Cyr and Robaire, 1992; Tenniswood et al., 1998) and to be similar in molecular weight to the Sertoli-derived SGP-2 except for variations in glycosylation (Sylvester et al., 1984). In the caput, corpus and proximal cauda epididymal regions, a checkerboard staining pattern was observed in the case of principal cells with cells alongside each other being either intensely, moderately or weakly reactive, suggesting that cells were out of synchrony with respect to the synthesis and secretion of SGP-2 (Hermo et al., 1991). In the epididymal lumen, a major fraction of SGP-2 was free or loosely associated with sperm, whereas a smaller fraction was more tightly bound (Law and Griswold, 1994). It has been suggested that SGP-2 provides the sperm surface with a protective coat guarding against non-specific proteases, glycosidases or other injurious agents or that it solubilizes and transports lipids from principal cells to the sperm surface (Hermo et al., 1991; Law and Griswold, 1994), although many other functions have been attributed to SGP-2 (Tenniswood et al., 1998). Found in many other tissues, SGP-2 is associated with numerous physiological and pathological conditions (Jenne and Tschopp, 1992).

In the present study, SGP-2 was localized within principal cells of the cauda epididymidis in a checkerboard staining pattern like that described in the rest of the

epididymis (Table 2, Fig. 8b). However, along the entire vas deferens, the cytoplasm of all principal cells was intensely and uniformly reactive (Table 2, Fig. 8b). These data thus indicate region specific differences in the expression of SGP-2 by principal cells of these regions, the functional significance of which is unclear. The cytoplasmic reaction in principal cells as seen in the present study was shown by EM analysis in the epididymis to be indicative of SGP-2 secretion into the lumen (Hermo et al., 1991), suggesting a similar activity for principal cells in the vas deferens. Indeed, in the present study, a reaction was also noted in association with sperm. Thus, SGP-2 may function with the regard to sperm during their storage in these regions. In the cauda and proximal vas deferens other proteins, designated as HIS 50 and HIS 100, are secreted into the lumen where they bind to sperm (Rifkin and Olson, 1985). Differences in the molecular weight and immunostaining pattern of these two proteins indicate that they differ from SGP-2, but that other proteins are also secreted by these regions which function with regard to sperm.

Low density lipoprotein receptor related protein-2 (LRP-2), also known as gp330 or megalin, is a member of a family of endocytic receptors related to the low density lipoprotein receptor, all of which mediate cellular internalization and lysosomal degradation of various ligands (Kounnas et al., 1993, 1994). LRP-2 is highly expressed in coated pits, vesicles and endosomes of various epithelial cells and has been shown to bind SGP-2 with high affinity (Kerjaschki and Farquhar, 1982; Kounnas et al., 1995; Zheng et al., 1994). In the epididymis, LRP-2 was localized to the apical surface of principal cells where it was suggested to mediate the uptake of SGP-2 (Morales et al., 1996). Together with the finding that principal cells of the intermediate zone not only secreted but endocytosed SGP-2, it was suggested that SGP-2 may be actively turned over in the epididymal lumen after performing its function(s) with sperm (Hermo, 1995).

In the present study, LRP-2 was expressed along the apical surface of principal cells of the cauda and vas deferens (Table 2, Fig. 8b). As SGP-2 appears to be secreted by principal cells of the cauda and vas deferens, it could be suggested that these cells express LRP-2 to remove SGP-2 from the lumen. Thus, as in the case of the epididymis, SGP-2 would be available to sperm in the lumen of the cauda and vas deferens and after performing its function would be internalized by LRP-2 and eventually degraded in the lysosomes of principal cells, indicating an active turnover of SGP-2 in the lumen. Furthermore, as in the

epididymis, clear cells were unreactive indicating no role for these active endocytotic cells in the uptake of SGP-2.

In summary, the present study reveals cell and region specific expression of various lysosomal proteins in the cauda epididymidis and vas deferens suggesting substrate specificity with respect to the substances internalized by these cells. In addition, principal cells of the cauda and vas deferens secrete SGP-2 into the lumen where it associates with sperm. In addition, LRP-2 is expressed on the apical surface of principal cells suggesting that SGP-2 is internalized by principal cells after performing its function in the lumen in relation to sperm.

4.7. ACKNOWLEDGMENTS:

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CHAPTER FIVE:
SUMMARY AND CONCLUSION

As reviewed in the first chapter, principal cells show marked structural differences in the proximal, middle, and distal regions of the vas deferens reflective of diverse functional activities. In this thesis, their structural features were examined in the electron microscope of glutaraldehyde-fixed, Epon-embedded material, while functional parameters were examined by light microscope immunocytochemistry of Bouin-fixed, paraffin-embedded material.

The structural features of epithelial cells of the rat vas deferens was studied in the second chapter. It was found that in the proximal region, the cuboidal principal cells resembled those of the cauda epididymidis but few clear cells and occasional narrow cells were present. In the middle region, principal cells often contained blebs of their apical cytoplasm containing vesicular and tubular profiles. These blebs extended far from the cell surface and appeared to be liberated into the lumen suggesting an apocrine type of secretion. In the distal region, dilated intercellular spaces containing numerous membranous profiles of different shapes and sizes were noted between adjacent principal cells and overlying basal cells. The infranuclear cytoplasm of principal cells of this region showed whorls of sER. Large intracytoplasmic cavities were found within the sER aggregates and contained membranous profiles which appeared to peel off from the surrounding sER elements. Various images of such cavities closely juxtaposed to the lateral plasma membrane suggested that the membranous profiles of the intercellular spaces were derived from them.

The first function studied in the second chapter was water transport. Anti-AQP-1 antibody revealed an intense reaction over the endothelial cells of numerous vascular channels in the lamina propria and this, taken together with morphological features described above, suggested water transport from the lumen of the vas deferens via the dilated spaces to underlying vascular channels, the function of which may be to concentrate sperm.

The second function studied in the second chapter was steroid synthesis. Anti-3β-HSD antibody revealed an intense reaction over principal cells of the vas deferens, as well as over the blebs in the vas deferens lumen, and this was taken as evidence for steroid synthesis by these cells. Moreover, it was suggested that the release of sER membranous profiles into the dilated spaces and presence of blebs in the lumen may represent a means of

transporting steroids out of principal cells destined for different sites. Steroids in the blebs would be destined ultimately for utilization by luminal sperm, while that of the dilated spaces for muscle layers of the lamina propria.

In the third chapter, the ability of the vas deferens to create a special environment for protecting spermatozoa in the lumen was investigated using anti-GST antibodies. The distribution of Ya , Yb1, Yc, Yf and Yo subunits was examined. Both principal and basal cells showed varying degrees of GST expression in the different regions of the cauda and vas deferens, suggesting a complex, changing environment of substrates to which these cells are subjected. Furthermore, while expression often differs between principal and basal cells, the absence of reactivity of a given GST in one cell type is usually compensated for by expression in the other cell type in any given region of the cauda or vas deferens.

In the fourth chapter, the expression of cathepsins A, D and B, SGP-1 as well as LRP-2 was examined to study endocytic functions of epithelial cells lining the cauda epididymis and vas deferens. In addition, SGP-2 expression was examined to study the secretory functions of these cells. The expression of different lysosomal enzymes in the cauda epididymidis and vas deferens was not only cell but also region specific suggesting differences in the type of substrates internalized by these cells. The cell and region specific expression of SGP-2 and LRP-2 suggested that SGP-2 is involved in active turnover, where it is not only secreted but endocytosed by principal cells of cauda epididymidis and vas deferens.

In summary, epithelial cells of the rat vas deferens appeared to be involved in 1) eliminating water from the vas deferens lumen, 2) synthesis and secretion of steroids, 3) providing ample protection of sperm from harmful circulating electrophiles during their storage and 4) synthesis and secretion of proteins and endocytosis and breakdown in lysosomes of other proteins. All of these functions must aid in sperm maintenance and protection while they are stored in this region.

CHAPTER SIX:
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