In presenting this thesis in partial fulfilment of the requirements for an advanced
degree at the University of British Columbia, I agree that the Library shall make it
freely available for reference and study. I further agree that permission for extensive
copying of this thesis for scholarly purposes may be granted by the head of my
department or by his or her representatives. It is understood that copying or
publication of this thesis for financial gain shall not be allowed without my written
permission.
The cytoskeleton is a prominent component of mammalian Sertoli cells and is thought to play a major role in the dramatic changes that Sertoli cells undergo during the spermatogenesis. Actin filaments as well as microtubules are found in areas of Sertoli cells which are intimately related to the developing germ cells. In addition, actin filaments and microtubules in the ground squirrel (*Spermophilus lateralis*) change their distribution during the stages of germ cell development (Vogl & Soucy, 1985; Amlani & Vogl, 1986; Vogl & Amlani, 1987). One objective of this study was to determine whether intermediate filaments, the third component of the cytoskeleton, change their distribution during the spermatogenic cycle of the rat and of the ground squirrel, and if so, to determine whether these changes correlate with changes in the germ cell population.

The distribution of vimentin intermediate filaments in Sertoli cells during spermatogenesis was observed with immunofluorescence, and was confirmed with electron microscopy.

The distribution of vimentin filaments within Sertoli cells changes with changes in the germ cell population. At stages where elongate spermatids reside in crypts located deep within the seminiferous epithelium, groups of eight to twelve intermediate filaments were consistently found at the convex surface of the spermatid heads. Here the filaments are in close association with ectoplasmic specializations. At later stages of spermatogenesis, intermediate filaments are not found in crypt areas. Because of their
association with particular stages of developing germ cells, intermediate filaments in Sertoli cells may be involved in the attachment and positioning of developing germ cells within the seminiferous epithelium.

Intermediate filaments in general are thought to be involved in anchoring the nucleus and cytoplasmic organelles within a cell. In order to test this hypothesis, acrylamide, a specific perturbant of intermediate filaments in vitro, was perfused through rat and ground squirrel testes in order to perturbate the intermediate filament system within Sertoli cells. No effects of acrylamide on intermediate filaments were observed in vivo at either the light microscopic or ultrastructural level. However, toxic effects were observed upon treatment with high concentrations of acrylamide, indicating that Sertoli cells and associated germ cells were indeed exposed to the perturbant.

Based on these studies, one can conclude that: (1) vimentin filaments in Sertoli cells change their distribution during spermatogenesis; (2) vimentin filaments are closely associated with specific stages of developing germ cells, and may be involved in the positioning and attachment of these cells to Sertoli cells within the seminiferous epithelium, and (3) acrylamide has no effect on vimentin filaments in Sertoli cells in vivo.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1. Sertoli cells</td>
<td>2</td>
</tr>
<tr>
<td>2. Cytoskeleton in Sertoli cells</td>
<td>3</td>
</tr>
<tr>
<td>a) Actin Filaments</td>
<td>3</td>
</tr>
<tr>
<td>b) Microtubules</td>
<td>3</td>
</tr>
<tr>
<td>c) Intermediate Filaments</td>
<td>4</td>
</tr>
<tr>
<td>THESIS TOPIC</td>
<td>25</td>
</tr>
<tr>
<td>1. Intermediate Filaments in Sertoli Cells - Background</td>
<td>27</td>
</tr>
<tr>
<td>2. Thesis Project</td>
<td>28</td>
</tr>
<tr>
<td>3. Animal Models</td>
<td>33</td>
</tr>
<tr>
<td>DISTRIBUTION OF INTERMEDIATE FILAMENTS DURING SPERMATOGENESIS</td>
<td>35</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>35</td>
</tr>
<tr>
<td>2. Materials and Methods</td>
<td>37</td>
</tr>
<tr>
<td>a) Immunofluorescence</td>
<td>37</td>
</tr>
<tr>
<td>b) Homogenization of Testicular Tissue</td>
<td>38</td>
</tr>
<tr>
<td>c) Immunoblot</td>
<td>38</td>
</tr>
<tr>
<td>d) Electron Microscopy</td>
<td>39</td>
</tr>
<tr>
<td>3. Results</td>
<td>40</td>
</tr>
<tr>
<td>4. Discussion</td>
<td>61</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>PERTURBATION OF INTERMEDIATE FILAMENTS AS A MEANS TO STUDY FUNCTION</td>
<td>64</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>64</td>
</tr>
<tr>
<td>2. Materials and Methods</td>
<td>68</td>
</tr>
<tr>
<td>a) Perfusion of Testes with Acrylamide</td>
<td>68</td>
</tr>
<tr>
<td>3. Results</td>
<td>68</td>
</tr>
<tr>
<td>4. Discussion</td>
<td>89</td>
</tr>
<tr>
<td>SUMMARY AND FUTURE CONSIDERATIONS</td>
<td>92</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>95</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Figure 1</td>
<td>Structure of an IF protofilament.</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Structure of a Type III IF.</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Mechanisms of IF attachment to the plasma membrane.</td>
</tr>
<tr>
<td>Figure 4</td>
<td>General distribution of IFs in Sertoli cells of the rat ground squirrel.</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Relationship between IFs in different regions of the Sertoli cell.</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Distribution of vimentin IFs in rat Sertoli cells during spermatogenesis as illustrated by immunofluorescence.</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Distribution of vimentin IFs in ground squirrel Sertoli cells, as illustrated by immunofluorescence.</td>
</tr>
<tr>
<td>Figure 8</td>
<td>SDS-PAGE gels and immunoblots of testicular homogenates.</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Immunofluorescence controls for secondary antibody specificity and for autofluorescence.</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Electron micrograph of IFs in rat Sertoli cell crypts at an early stage of spermatogenesis.</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Electron micrographs of IFs in rat Sertoli cell crypts at an early stage of spermatogenesis.</td>
</tr>
</tbody>
</table>
Figure 12. Electron micrographs of rat Sertoli cell crypts at a late stage of spermatogenesis. 58
Figure 13. Electron micrographs of IFs in ground squirrel Sertoli cell stalks. 60
Figure 14. Schematic diagram of the perfusion apparatus utilized in IF perturbation experiments. 70
Figure 15. Light micrographs of seminiferous tubules after treatment with 5mM acrylamide. 74
Figure 16. Electron micrographs of Sertoli cell regions after treatment with 5mM acrylamide. 76
Figure 17. Light micrographs of seminiferous tubules after treatment with 10mM- and 50mM acrylamide. 78
Figure 18. Electron micrographs of Sertoli cell regions after treatment with 10mM acrylamide. 80
Figure 19. Electron micrographs of Sertoli cell regions after treatment with 50mM acrylamide. 82
Figure 20. Light micrographs of seminiferous tubules after treatment with 100mM acrylamide. 84
Figure 21. Electron micrographs of Sertoli cell regions after treatment with 100mM acrylamide. 86
Figure 22. Electron micrographs of toxic effects within Sertoli cells with 100mM acrylamide. 88
ACKNOWLEDGEMENTS

I would like to express my gratitude:

To my supervisor, Dr. A. Wayne Vogl, not only for his enthusiasm and guidance throughout the course of this investigation, but also for his training in the scientific approach;

To Dr. Auersperg, Dr. Emerman, and Dr. Gosline, members of my graduate committee, for their interest and helpful suggestions;

To Mr. Bruce C. Stewart for his artwork;

To Mrs. Darlene Redenbach and Dr. Bryon Grove for stimulating discussions and company at odd hours;

And to Mr. Ed Clemens for all his patience, support, and constant encouragement.

This work was supported by a grant awarded to Dr. A. W. Vogl by the Medical Research Council of Canada; Grant # MA-8020.
INTRODUCTION

Seminiferous tubules, housed in the testes, are the site of spermatogenesis: the development of haploid spermatozoa from diploid spermatogonia. Sertoli cells, along with spermatogenic cells, make up the epithelial portion of the seminiferous tubule and play an important role in spermatogenesis. Among numerous other functions, Sertoli cells provide structural support, nutrients, and other substances (Parvinen et al., 1982; Wright et al., 1982) essential to the proper development of spermatogenic cells. The developing germ cells are found in intimate contact with Sertoli cells; that is, they are embedded within cytoplasmic infoldings along the lateral surfaces of Sertoli cells, with immature spermatogonia residing in the basal compartment below the junctional complexes between Sertoli cells, while more differentiated spermatogenic cells can be found within lateral infoldings and crypts in the adluminal compartment above the junctional complexes (Ross & Reith, 1985; Dym & Fawcett, 1970). During the course of sperm development, germ cells continually change their relationship with Sertoli cells, but the two cell types are always found in close association (Russell, 1984).

The cytoskeleton of Sertoli cells appears to play a role in the process of spermatogenesis. Vogl and coworkers (Vogl et al., 1983; Vogl et al., 1983; Vogl & Soucy, 1985); have observed that the distribution of microtubules and microfilaments (actin filaments) change with the stages of spermatogenesis. Whether or not the distribution of intermediate filaments changes during spermatogenesis has not yet been determined.
Sertoli Cells

Sertoli cells are epithelial cells of mesenchymal origin found in seminiferous tubules within the testes. They are columnar cells which extend from the basal lamina overlying the flattened myoid cells and connective tissue to the lumen of the tubule. They have a basally located nucleus containing a prominent nucleolus which is flanked by DNA-containing karyosomes. The significance of these karyosomes is not known. The lateral surfaces of the Sertoli cells have many infoldings that are closely associated with spermatogenic cells at different stages of development. Sertoli cells contain a large amount of smooth endoplasmic reticulum (SER), many mitochondria, some rough endoplasmic reticulum (RER), lysosomes, as well as a well developed Golgi apparatus, although few secretory vesicles have been observed (Fawcett, 1975). Unlike other epithelial cells, junctional complexes of Sertoli cells – consisting of tight junctions, adherens junctions, and gap junctions – are found near the bases of the Sertoli cells. Tight junctions between Sertoli cells function as the "blood-testis" barrier (Dym & Fawcett, 1970) and serve to compartmentalize the epithelium, thus allowing for differences in fluid composition between the basal and adluminal compartments. The formation of distinct microenvironments appears to be essential for the proper development of germ cells (Ploen & Ritzen, 1984). Always seen in association with tight junctions are ectoplasmic specializations (Russell, 1977; Russell et. al., 1980), consisting of bundles of actin in hexagonal array sandwiched between the plasma membrane of a Sertoli cell and a cisterna of smooth endoplasmic reticulum. These ectoplasmic specializations have been observed between Sertoli cells and between Sertoli cells and germ cells, although in the latter case the specialization occurs only on the Sertoli cell side.
Each component of the elaborate cytoskeleton of Sertoli cells appears to play some role in organizing the spermatogenic cells during spermatogenesis. Microfilaments, composed of filamentous actin, 5 to 8 nanometres in diameter, are concentrated in ectoplasmic specializations (Toyama, 1976; Russell, 1977; Vogl & Soucy, 1985), and electron microscopy and NBD-phallacidin staining indicate that these filaments change their distribution during spermatogenesis (Vogl & Soucy, 1985). In the ground squirrel, actin filaments cannot be detected in association with round spermatids, but do appear as the round spermatids begin to elongate. Here, actin filaments can be observed in the region surrounding the nucleus of the spermatid capped by the developing acrosome. When the spermatid is fully elongated, actin bundles cover the spade-shaped head of spermatids; on one side of the head these actin bundles are arranged perpendicular to the long axis of spermatids, and on the other side they are arranged parallel to the long axis of spermatids on the other side of the head. As elongate spermatids undergo further maturation, the actin bundles take on a spiral arrangement, and are observed around the rim of the spermatid head. The actin bundles then become less abundant, and finally disappear at spermiation. Because actin filaments are found in ectoplasmic specializations, and ectoplasmic specializations are morphologically associated with sites of adhesion to spermatids, it has been suggested that the actin may be involved in intercellular attachment of spermatids to Sertoli cell crypts (Dym & Fawcett, 1970; Russell, 1977; Toyama, 1976; Vogl & Soucy, 1985). They have also been suggested to maintain the shape of crypts (Russell, 1977; Vogl & Soucy, 1985), and to be involved in spermiation (Fawcett, 1975; Toyama, 1976).

Microtubules (Mts) are 24 nanometres in diameter, are composed of the globular protein tubulin, and are the basic units of cilia, flagella, and
centrioles. They are known to form the mitotic spindle during cell division and are of importance in axoplasmic flow in neurons (Weber & Osborn, 1982). In ground squirrel Sertoli cells (Vogl et al., 1983; Vogl et al., 1983, Amlani & Vogl, 1986; Vogl & Amlani, 1987) microtubules are found in the apical cytoplasm parallel to the long axis of the cell as described in other Sertoli cells by Christensen (1965) and Fawcett (1975) in Sertoli cell stalks which support elongate spermatids, in cytoplasmic processes which extend into the residual cytoplasm of spermatids, and adjacent to the concave surface of the acrosome of late spermatids. In this latter area Mts form C-shaped structures which are discontinuous with the linear tracts of MTs in stalks, although they appear to be derived from these. These structures disappear at spermiation. Because Mts are seen in areas involved in the movement of spermatids and in areas of accumulation of SER, it has been suggested that they may be involved in translocating spermatids (Russell, 1977); in withdrawing residual cytoplasm from late spermatids; in shaping the developing acrosome (Vogl et al., 1983); and in transporting organelles within the cell.

Intermediate Filaments in General

Intermediate filaments (Ifs) are highly stable filaments composed of a chemically heterogeneous group of subunits. Based on the immunological and biochemical properties of these subunits, IFs have been placed into five classes (Anderton, 1981; Lazarides, 1980; Steinert et al., 1984; Zackroff et al., 1984): a) keratin filaments, found in epithelial cells; b) vimentin filaments found in mesenchymally derived cells; c) desmin or skeletin filaments, found in muscle; d) neurofilaments, found in neurons; and e) glial filaments, found in glial cells. Unlike microtubules and microfilaments little is known about the functions of intermediate filaments in general,
although numerous functions including positioning of organelles within the cytoplasm (Eckert, 1985; Fey et al., 1984), cell-cell adhesion (Jones & Goldman, 1986), cell shape (Steinert et al., 1984), endocytosis/exocytosis, cell division (Franke, et al., 1984), cell migration, and even a possible involvement with generation of energy in non-muscle cells (Eckert et al., 1980) have been suggested.

Over the past few years a great deal of research has been done on each one of the classes of IFs. The following is a brief review of each of class.

**Keratin Filaments**

Keratin filaments are found in many epithelial tissues and in epithelially-derived tissues. They are actively synthesized by the epidermis and are the major differentiation products of this tissue. The outer stratum corneum of the epidermis consists mainly of keratin filaments and associated proteins (Lazarides, 1984). Using electron microscopy, the keratin filaments are seen attached to desmosomes along the lateral surfaces of epithelial cells. Immunofluorescence techniques have been used to localize keratin filaments within epithelial cells, and also to identify cells in culture or in tissue sections as epithelial (Franke et al., 1978a,b; Franke et al., 1979). Many investigators (Cabral et al., 1980; Franke et al., 1978b; Franke et al., 1979) have observed that keratin filaments, stained with anti-keratin antibody and visualized by a fluorescent tag, form a network which extends throughout the cytoplasm and also envelopes the nucleus. Jones et al. (1982) state that the keratin filaments appear to be in continuous bundles running from the plasma membrane to the nuclear membrane and are associated with nuclear pores. Fey et al. (1984), using embeddment-free sections of Madin-Darby canine kidney (MDCK) cells observed that at the
ultrastructural level keratin filaments were localized at cell borders and were associated with the nuclei after removal of phospholipid, nucleic acids, and most of the cellular proteins. Fey and coworkers therefore suggest that IFs may act as a scaffold for the cytoskeletal framework. Jones et. al., 1985 have observed that a cage of keratin filaments in mouse keratinocytes forms around the mitotic spindle and are closely associate with the chromosomes throughout mitosis. They suggest that IFs may be involved in pole-to-pole separation of chromosomes.

Gel electrophoresis using 8M urea or other denaturing agents separates the keratin filaments into a family of polypeptides ranging from 40,000 to 65,000 daltons (Moll et. al., 1982). At present at least 20 keratin filament proteins have been established (Sun & Green, 1978). Simple epithelial cells lining ducts tend to express the smaller molecular weight proteins - an acidic 46,000 dalton protein and a basic 56,000 dalton protein (Steinert et. al., 1984; Sun et. al., 1979), for example, while more complex epithelia tend to express higher molecular weight polypeptides.

Keratin filament production is controlled by a multi-gene family, resulting in the activation of a particular subset depending on cell type, stage of differentiation, and organization of the epithelium. Basal cells of the epidermis produce low molecular weight polypeptides because they may be expressing a subset of genes activated in relatively undifferentiated epithelium, versus another subset which may be activated only in the subsequent more differentiated epithelial cell layers of the epidermis (Anderton, 1981). Glass et. al. (1985) have reported the presence of a mesothelial keratin which has uncharacteristically short amino and carboxy terminals for keratins, supporting the notion of activation of particular keratin genes depending on the type of cell and its function. Schmid et. al.
(1983) suggest that the presence of certain hormones may influence the expression of keratin filament proteins in bovine mammary gland in that cells grown in the absence of insulin, hydrocortisone, and prolactin contained a different set of keratins in that two acidic proteins of molecular weights 50,000 and 45,000 were no longer present.

**Vimentin Filaments**

Vimentin filaments are the class of IFs found in mesenchymally derived cells. Like keratin, vimentin is highly insoluble. In fibroblast cultures these proteins - 58,000 daltons - are observed by immunofluorescence, immunoperoxidase staining, and stereo-electron microscopy to be associated with microtubules near the plasma membrane, and are said to envelope the nucleus in a "cage-like" structure (Henderson & Weber, 1980). Cabral et. al. (1980) have observed that the vimentin filaments radiate from the perinuclear region to the periphery of the cell. When vimentin-containing cells are treated with microtubule inhibitors such as colchicine the vimentin filaments aggregate into bundles, forming a perinuclear cap, and redispere upon removal of the drug (Henderson & Weber, 1980; Starger et. al., 1978). It was this response to colchicine treatment that had lead to the belief that IFs were disaggregation products of microtubules. A transient perinuclear cap was also observed by Starger et. al., 1978 after cell division and spreading of BHK-21 cells and also after replating of the cells; however the significance of this behavior has not been established as yet. During mitosis in vascular endothelial cells vimentin filaments undergo several alterations. At interphase a sphere of vimentin filaments surrounds the nucleus. During prophase and metaphase, the sphere becomes wavy due to rounding up of the cells. The vimentin network then alters its shape during anaphase to become
rectangular and encompasses the chromosomes and spindle apparatus. The rectangle is cleaved into two crescents at telophase which subsequently re-organize into spheres in the daughter cells (Blose, 1979). Franke et. al. (1984), Aubin et. al. (1980) and Jones et. al. (1985) observe similar rearrangements of vimentin filaments during mitosis in several cell lines including bovine kidney epithelial cells, rat kangaroo PtK2 cells, and HeLa cells, respectively. In the latter cell line, the co-existing keratin filaments form amorphous non-filamentous bodies leading Jones et. al. (1985) to suggest that an IF system consisting of only one major structural protein—as in the case with vimentin—may be divided in a more simple fashion between the resulting daughter cells than the keratin filament system, which consists of many structural proteins.

Desmin Filaments

Desmin filaments are found in smooth as well as striated muscle, but are most abundant in smooth muscle. Desmin filaments are composed of an acidic 55,000 dalton protein which has two isotonic variants depending on the amount of phosphorylation (Steinert et. al., 1984). These filaments are thought to form a cytoskeleton that holds the myofibrils within muscle cells in place. Desmin is localized to the periphery of Z-discs in both skeletal and cardiac muscle, and is also localized to cardiac intercalated discs. They also found that these filaments linked Z-discs to one another in a honeycomb arrangement, thus supporting the theory that desmin forms a network between myofibrils in a muscle fibre. Behrendt, 1977 also localized desmin at Z-discs, but found desmin completely surrounded the Z-discs and connected the discs to muscle triads formed at the Z-line. In smooth muscle desmin filaments form a network which links cytoplasmic dense bodies with membrane bound dense bodies. The
dense bodies appear to be analogous to Z/discs of skeletal muscle (Lazarides, 1980). Although desmin has been established as the class of intermediate filaments characteristic of muscle cells, Frank & Warren (1981) report that smooth muscle cells of the aorta contain vimentin instead of desmin when analysed by immunofluorescence and gel electrophoresis. Since then several investigators including Granger & Lazarides (1979) have localized both desmin and vimentin filaments in muscle cells.

Neurofilaments and Glial Filaments

Neurofilaments and glial filaments are found within cells of the nervous system. In neurons IFs appear to be distributed throughout the neuron and in association with microtubules along the axes of the cell processes (Steinert et. al., 1980). Unlike other IF classes, native and in-vitro assembled neurofilaments appear to be linked to each other by side arms (Lazarides, 1980; Steinert et. al., 1980). Willard & Simon (1981) suggest that these side arms may function in mediating interactions between adjacent neurofilaments and also between neurofilaments and other cytoplasmic structures such as microtubules. Biochemical analysis reveals that neurofilaments are composed of three polypeptides (the neurofilament triplet) of 200,000, 150,000, and 68-70,000 daltons, with the latter protein being the backbone of the filament, the 200,000 dalton protein localized more peripherally and repeated every 100nm, and the 150,000 dalton protein localized along the backbone (Sharp et. al., 1982). Sharp et. al., 1982 report that certain neurons on dorsal root ganglia do not stain with anti-neurofilament antibody, while others stain quite heavily, suggesting that neurofilaments may not be essential to the functioning of neurons, or that neurons that do not appear to contain neurofilaments may perform a function yet unknown.
Glial filaments are composed of glial fibrillar acidic protein of molecular weight 51,000 daltons. These filaments are reported to be dispersed throughout the cytoplasm of glial cells such as astrocytes (Lazarides, 1980).

Co-existence of Multiple Intermediate Filaments Types in a Single Cell

Although in general, a particular cell type contains only one class of intermediate filaments, several laboratories have reported the co-existence of two types of intermediate filaments within the same cell (Frank et al., 1982; Gard et al., 1979; Granger & Lazarides, 1979; Henderson & Weber, 1981; Lane et al., 1983; Schmid et al., 1983). Franke et al. (1978, 1979) have found that HeLa and PtK2 cell lines contain keratin filaments as well as vimentin where perinuclear aggregates of vimentin can be visualized in these epithelial cells by immunofluorescence after treatment with colchicine. Existence of both types of intermediate filaments within a cell has been verified by gel electrophoresis. Similarly, both vimentin and desmin have been localized in BHK-21/C13 cells by double immunofluorescence studies (Frank et al., 1982). The two IF subunits appear to co-exist within the same IF structure as a co-polymer. To ensure that the co-expression of vimentin and desmin within the same cell is not a result of cell culture conditions, Frank et al. (1982) examined baby hamster kidney (BHK) cells—frozen sections as well as cell preparations—and found that both IF classes were expressed in vivo as well. The investigators found that antibodies to desmin bound to tissue in the region of the mesangial cells at the vascular pole of the kidney, supporting the theory that mesangial cells are undifferentiated smooth muscle cells. The co-localization of desmin and vimentin at Z-discs of striated muscle fibres, forming a honeycomb network within each Z plane is more evidence that
co-expression of IFs is not a condition of tissue culture. In addition, it is well accepted that both desmin and vimentin co-exist in embryonic myotubes, and these filaments are thought to co-ordinate the formation of myofibrils in developing muscle (Lazarides, 1982).

Intermediate Filaments Associated With Desmosomes

Intermediate filaments are always found in association with desmosome junctions (Farquar & Palade, 1963). These junctions occur at the lateral aspects of epithelial cells and serve to adhere neighboring cells to one another. Ultrastructurally, desmosomes (macula adherens) are composed of two dense plaques located on the cytoplasmic side of each cell involved in forming the junction. It is into these plaques that intermediate filaments attach (Franke et al., 1981; Kartenbeck et al., 1983; Kartenbeck et al., 1984; Jones et al., 1982, Drochmans et al., 1978; Schwechheimer et al., 1984). The plaques are separated by an intercellular space of approximately 20 nanometres. Within this space is an electron dense material which runs the length of the desmosomal junction.

The composition of desmosomes has been determined by isolating desmosomes from bovine muzzle epidermis (Kapprell et al., 1985; Cowin et al., 1985; Drochmans et al., 1978; Gorbsky & Steinberg, 1981) and then electrophoretically separating the components. The intercellular region or "core" is composed of at least three glycoproteins termed "desmogleons" (Gorbsky & Steinber, 1981; Cohen et al., 1983; Kapprell et al., 1985; Cowin et al., 1985). The membrane proteins in the desmosome region are termed "desmocollins". At least four proteins have been localized to the plaque regions of the desmosome (Cowin et al., 1985; Kapprell et al., 1985; Franke
et. al., 1981; Mueller & Franke, 1983). The two higher molecular weight proteins, desmoplakins I and II, are thought to be the points of attachment of IFs to the desmosome complex (Gigi-Leitner & Geiger, 1986). There is some similarity in amino acid composition between desmoplakins and keratins as well as other IF-associated proteins such as filaggrins; these glycine-rich areas may be responsible for bundling keratins at the desmosomes (Cohen et. al., 1983; Kapprell et. al., 1985; Gigi-Leitner & Geiger, 1986).

Desmosomes in different epithelia – simple stratified, pseudostratified – as well as in different tissues, such as myocardium or meninges, have very similar plaque components, even though the junctions may differ in size, in substructure, and even in the class of IFs attached to the desmoplakins (Cowin et. al, 1985; Franke et. al., 1982; Kartenbeck et. al., 1983; Kartenbeck et. al., 1984a,b). Desmosomes found in intercalated discs of cardiac muscle, as well as those found in Purkinje fibres of the heart, have attached to them IFs composed of desmin (Kartenbeck et. al., 1983). IFs attached to desmosomes in arachnoid tissue are composed of vimentin (Kartenbeck et. al., 1984ab).

The two major components in this type of adhering junction are the desmosome proper and the intermediate filaments. The assembly of desmosomes has been studied by several laboratories to determine whether plaque assembly is essential for IF attachment or vice versa. There is general agreement that desmosome formation and IF attachment requires cell-to-cell contact and the presence of calcium. Cells grown in culture media containing low calcium levels (0.1mM) have little contact between cells and do not contain desmosomes (Watt et. al., 1984; Denefle & Lechaire, 1986, Jones & Goldman, 1985; Bologna et. al., 1986). Also at low calcium concentrations, desmosomal core glycoproteins are distributed over the entire cell surface, whereas the plaque proteins are distributed throughout the cytoplasm (Watt et. al., 1984) or are
distributed in the perinuclear region of the cell (Jones & Goldman, 1985). In addition, keratin filaments are located in a perinuclear region and do not extend to the periphery of the cells (Jones & Goldman, 1985; Watt et. al., 1984). However, Bologna et. al., 1986 claim that keratin filaments are not found in the juxtanuclear region, but in fact disappear under low calcium conditions. When calcium concentrations are increased (1.2 - 1.8mM), desmosome plaque proteins as well as desmosome core glycoproteins become more concentrated at the cell surface, in areas of cell to cell contact. Concomitantly, keratin filament bundles change their distribution from a perinuclear one to one that extends throughout the cytoplasm to areas of cell to cell contact (Jones & Goldman, 1985; Watt et. al., 1984).

Based on these studies, it appears that desmosomal components are preformed and distributed in various regions of the cell. The assembly of these components into desmosomes requires cell contact as well as adequate calcium levels. The accumulation of desmosome components at sites of cell contact is not a pre-requisite for the re-organization of intermediate filaments; that is, intermediate filaments re-organize within a cell regardless of the location of desmosome components. Although the cells in which desmosome assembly has been studied contain keratin filaments it is reasonable to assume that desmosome formation in cells containing vimentin or desmin filaments may be similar; the complement of desmosomal proteins is almost identical and the IFs - regardless of their composition - attach to desmoplakin I.

Intermediate Filament Substructure
The five classes of intermediate filaments are composed of subunits which differ in molecular weight and immunological properties, but all five classes
share a similar basic structure. The basic structure of the IF subunit has almost been illucidated and there is a growing amount of information about the genes coding for these filaments and factors governing the expression of these genes.

With respect to IF structure, each subunit is composed of a central alpha-helical rod domain flanked by globular end domains. A protofilament is composed of two IF subunits. The rod domain of a subunit is made up of 311 to 314 amino acids, and has a length of 45 to 48nm (Steinert et. al., 1983; Crewther et. al., 1983; Fraser & McRae, 1973) and a highly conserved secondary structure. It consists of two alpha-helices which form a coiled-coil when they come together (Crewther et. al., 1978) (Fig 1) due to its amino acid sequence of a discontinuous succession of heptads (abcdefg)n, where amino acids "a" and "g" are apolar. This gives rise to an inclined stripe of apolar residues which runs throughout the helix. The rod domain is divided into four subsegments: 1A, 1B, 2A, 2B, each of which are separated by short linker regions L1, L12, L2. L1 links 1A to 1B, L12 links subsegment 1 to subsegment 2, and L2 links 2A to 2B. These linkers do not form alpha-helices, but have the potential to form beta-sheets.

There are four types of IF subunits, each consisting of an alpha-helical coiled-coil rod domain and two end domains. Type I subunit is found in acidic keratin filaments, Type II subunit is found in neutral-to-basic keratins, Type III subunit in vimentin, GFAP, and desmin, and Type IV subunit is found in neurofilament proteins. Types I and III contain acidic 2B subsegments, while Types II and IV contain neutral 2B subsegments. The types of IF subunits differ with respect to their end domains. Both subunits that make up keratin filaments have end domains which are further divided into subdomains, V1 and V2 being variable in size. High molecular weight keratins have up to 130
Figure 1. Schematic illustration of two intermediate filament subunits, comprising an IF protofilament. Each protofilament consists of two polypeptides which come together to form a coiled-coil central rod domain flanked by globular head and tail regions. The globular domains are thought to be located on the periphery of the rod.
amino acid residues in their V subdomains, while low molecular weight keratins may even lack V subdomains. These V subdomains contain up to nine tandem repeats rich in glycine and/or serine. The amino and carboxy terminals are usually basic and highly variable. Type III subunits on the other hand consist of two homologous subdomains H1 and H2, where the H2 subdomain comprises the entire carboxy terminal. The remainder of the amino terminal, apart from H1, consists of strongly basic, hypervariable sequences of variable length. The composition of neurofilament (Type IV) end domains again differs from that of the other Types in that their amino terminal domain contains a variable region V, and their carboxy terminal domain contains a glutamic acid rich variable V2. A schematic diagram of a Type III intermediate filament is illustrated in Figure 2.

Assembly of IFs from the respective subunits appears to involve two critical steps: 1) formation of the coiled-coils, and 2) formation of a 4-chain complex or a small polymer of 2-chain complexes. There is speculation that dimer formation may involve the highly conserved sequences in subsegments 1A and 2B. Ionic interactions between subsegments may be important in bringing together neighboring 2-chain complexes to form 4-chain complexes, and subsequently to form IFs. In support of this, the phase of acidic and basic subunits— that is, Types I and II— differs by 1800, where regions of acidic and basic amino acid residues form alternate stripes along the coiled-coil molecule (Steinert et. al., 1985 a,b). X-ray diffraction studies along with visualization of IFs with scanning-transmission electron microscopy suggest
Figure 2. Schematic diagram of a Type III intermediate filament. Each IF is thought to consist of 8 two-chain complexes, with the rod domains of each complex being entwined with one another and with the globular portions extending over the periphery of the filament.
that a given length of IF contains the same number of subunits: 32 subunits—presumably eight 4-chain coiled-coil complexes—per axial repeat of 47nm. In incompletely formed IFs an axial periodicity of 22nm is present, and corresponds to the length calculated for subsegments 1 and 2 of the rod domain.

The amino and carboxy terminals of the end domains are thought to be located on the surface of the IF and may project from the cylindrical core of the molecule. Steinert et. al., 1983 provided evidence for this by showing that the integrity of mouse keratin IF could be maintained even after proteolytic cleavage of more than 60% of glycine-rich portions of the end domains. Also, IFs have a dense core of 9 nanometres and are surrounded by a diffuse substance extending the diameter of IFs to approximately 15 nanometres (Steinert et. al., 1985), suggesting that the widened diameter may be due to the amino and carboxy terminals of these end domains. Sauk et. al., 1984 found that removal of end domains with chymostrypsin inhibits IF assembly by inhibiting lateral binding of protofilaments, inferring that the globular end domains do play some role in IF formation.

**Genetic Regulation of Intermediate Filaments**

With respect to genes coding for intermediate filaments, there appears to be only one copy of any IF gene per haploid genome, in general. Of four different IF genes sequenced each gene contains seven or eight introns, mainly in regions encoding the rod domain. The position of these introns seems to be highly conserved in that three introns occur in exactly the same position in all four of the sequenced genes, two introns occur near each end of the 2B subsegment, and two other introns occur in regions coding for the carboxy terminal end domains. It also appears that introns occur at the beginnings of
heptad coding regions (Krieg et. al., 1985). Interestingly, Lau et. al. (1984) found that a minimum of four heptads are required to form 2-chain coiled coils, and that the smallest exon encoding heptad repeat sequences for rod domains correspond to 28 residues, thus forming four heptads.

Several investigators (Steinert et. al., 1984; Kreig et. al., 1985; Lau et. al., 1984) have speculated that the IF gene from which the variation into five established classes of IFs arose encoded for a relatively simple polypeptide capable of forming an alpha-helical coiled coil. Similar mechanisms have been proposed for the genetics of immunoglobulin genes and collagen genes, among others. The appearance of the types of IF subunits and their subdomains may have been the result of gene amplification, loss of certain introns, and divergence of exons. In response to the need for IFs to perform more specialized functions – that is, functions particular to a given cell type – larger variable regions were incorporated into each end domain.

Factors regulating IF gene expression are not well understood, although there is agreement that it is regulated at the transcriptional level (Farrari et. al., 1986; Lillenbaum et. al., 1986). Ferrari et. al. (1986) found that expression of the human vimentin gene could be induced with mitogens even in the presence of cyclohexamide. The results of Bologna et. al. (1986) imply that protein synthesis is required for maintenance of the filament system since treatment of rat mammary cells in culture with cyclohexamide resulted in the disappearance of existing keratin filaments; that is, there is not a pool of intermediate filament proteins within the cell. Bologna and co-workers also found that assembly of keratin filaments is dependent on cell-to-cell contact and formation of desmosome plaques since disassembly of desmosomes resulted in the disappearance of keratin filaments. Cell-to-cell contact may be a pre-requisite particular to IFs in epithelia requiring strong adhesion to
one another. HeLa cells have few desmosomes and appear to have their IFs
organized elsewhere in the cell.

**Intermediate Filament Attachment to Membranes**

Intermediate filaments form a wide network of filaments, which in most
cells, extends from the nuclear region throughout the cytoplasm to the
periphery of the cell. The mechanisms by which IFs attach to either the
nuclear membrane or the plasma membrane are not understood. Fey et. al.
(1984) found that vimentin IFs — after extraction of lipid, nucleic acids, and
other cytoskeletal elements — are associated with nuclear pores. Recent
reports (Georatos & Blobel, 1987a,b; Katsuma et. al., 1987) demonstrate the
attachment of vimentin filaments to the nuclear membrane. More specifically,
Georatos & Blobel (1987a,b) demonstrate the attachment of the carboxy tail
domain of vimentin to nuclear lamin B. With respect to binding of IFs to the
plasma membrane, the peripheral plasma membrane protein ankyrin binds vimentin
and also binds actin and spectrin to the plasma membrane. It appears that the
IF head piece (amino terminal domain) plays a role in the attachment of these
filaments to ankyrin since polymerization is inhibited upon the binding of
vimentin to isolated molecules of ankyrin (Georatos et. al., 1984). Langley
and Cohen (1986) put forth several models showing the possible interactions
that spectrin may have with actin and IFs in non-erythroid cells IFs are
connected in some way to the nuclear membrane, perhaps via nuclear pores, and
are connected to the plasma membrane via ankyrin. Ankyrin is bound to an
integral membrane protein, and also to spectrin. Spectrin is in turn bound to
intermediate filaments. Langley and Cohen also propose IFs are connected to
each other via spectrin molecules, and that spectrin may in fact link
different components of the cytoskeleton to each other (Figure 3).
Figure 3. A schematic diagram illustrating IF binding to the plasma membrane directly via ankyrin or indirectly via spectrin, which in turn binds to ankyrin. Spectrin may link IFs to each other or to actin filaments. (Modified from Georatos et al., 1984 and Langley & Cohen, 1986).
Changes in Intermediate Filaments During Development

It appears that cells in developing tissues are capable of changing the expression of one type of IF to another type at different stages of development (Franke et. al., 1982; Holthofer et. al., 1984). Cells which lose their cell-to-cell contact during embryogenesis change their expression of IFs from keratin to vimentin. Franke et. al. (1982) state that synthesis of vimentin begins in the primitive streak in primary mesenchyme cells and that there is a concomittant cessation of keratin production. The appearance of vimentin in mouse embryos correlates with the first appearance of non-epithelial cells. The mechanism by which the epithelial cells switch from their epithelial characteristics with respect to morphology, cytoskeleton organization, and desmosomes to mesenchymal cell characteristics has not been determined. Loss of epithelial cell continuity may be a factor in this conversion. Lane et. al. (1983) are in agreement with Franke's laboratory in that they also observed the expression of vimentin upon reduced cell-to-cell contact and detachment from the continuous epithelial cell layer. This observation was verified by the fact that cells at the free margins of stratifying colonies of primary keratinocyte cultures preferentially express vimentin. Differences in expression of IF within classes can also be seen. For example, embryonic epidermis expresses a different keratin from adult epidermis (Moll et. al., 1982), one that is similar to that found in simple epithelia. The expression of both vimentin and desmin in myotubes during myogenesis (Lazarides, 1982) appears to continue in the mature muscle fibre, and does not change like the expression of keratin and vimentin. The continual expression of vimentin may be a result of the mesenchymal origin of muscle tissue.
Intermediate Filament Function

Little is known with respect to the functions of intermediate filaments, because IFs are highly insoluble - more so than microtubules and microfilaments - it is likely that they are also functionally different. Some of the more accepted theories of IF function are: a) maintenance of cell shape (Eckert et al., 1984; Jones et al., 1982); b) spatial organization of cytoplasmic organelles (Eckert et al., 1985; Granger & Lazarides, 1979); and positioning or anchoring the nucleus (Henderson & Weber, 1980; Jones et al., 1982; Zackroff et al., 1984). The cage-like structure of keratin or vimentin around the nucleus strongly suggests that IFs are involved in the anchorage of the nucleus within the cytoplasm. Jones et al. (1985) state that the keratin-desmosome system is ideal for limiting distensibility of an individual cell because these filaments run from the perinuclear region to desmosomes at the periphery of the cell. Steinert et al. (1984) suggest that this array of IFs within the cell may also function as a means of transportation of information - electrical or mechanical - from the plasma membrane to the nuclear membrane. Behrendt (1977) proposed that in cardiac muscle IFs may mediate nucleo-sarcolemmal and nucleo-myofibrillar exchange. The association of creatin phosphokinase with IFs in cultured mammalian cells led Eckert et al. (1980) to suggest that IFs may be sites within the cytoplasm of non-muscle cells where enzymes involved in generation of energy are located. Creatine phosphokinase catalyses the formation of ATP from creatine phosphate and ADP in skeletal muscle.
Intermediate Filaments in Sertoli Cells

Intermediate filaments in Sertoli cells of the rat and ground squirrel as indicated by transmission electron microscopy are found at the base of the cell, as a layer adjacent to the plasma membrane (Figure 4a), around the nucleus (Figure 4d), in association with desmosome-like junctions between spermatogonia, spermatocytes and round spermatids (Figure 4c), and in the apical cytoplasm where they occur along with microtubules in Sertoli cell stalks which suspend late spermatids (Figure 4b), as well as in cytoplasmic processes which penetrate round and elongate spermatids (Franke et. al., 1979; Russell, 1977; Vogl et. al., 1983; Vogl et. al., 1985). The manner in which the intermediate filaments in each of these areas are related to one another is unclear (Figure 5). Are the IFs around the nucleus continuous with the filaments at the base of the cell and those in association with desmosome-like junctions? Also, how are the IFs in the apical cytoplasm related to those elsewhere in the cell?

Intermediate filaments in rat Sertoli cells have been characterized by Franke et. al. (1979) to be composed of vimentin. Intense fluorescence could be seen mainly in the basal cytoplasm with antibodies against vimentin. Although Sertoli cells constitute the epithelial component of the seminiferous epithelium, no fluorescence was detected using antibodies against keratins. Paranko et. al. (1986) have reported the co-existence of keratin and vimentin filaments in differentiating rat Sertoli cells. The keratin filaments disappear 14 days post-natally, while the vimentin filaments remain. Spruill et. al. (1983a,b) found that a 58,000 dalton protein was phosphorylated in a cAMP-dependent manner, in a calcium/calmodulin-dependent manner, and also in a
FSH-dependent manner. They identified this phosphoprotein as vimentin based on its molecular weight of 58,000 daltons, its isoelectric point of 5.1, and its similarity in peptide mapping on two-dimensional electrophoretic gels (Groppi & Browning, 1980). In addition, immunoprecipitation of a 58,000 dalton protein from 35[S]-methionine-labelled extracts occurred with antiserum against vimentin. These investigators have suggested that phosphorylated IFs may be involved in hormonal induction of an alteration in cell shape and in secretion of specific proteins in response to hormones.

Ultrastructurally, IFs can be seen in association with desmosome-like junctions in Sertoli cells. However, IFs normally associated with desmosomes are composed of keratins and are commonly known as tonofilaments. Therefore, the association of vimentin with desmosome-like junctions in these cells may be a unique situation.

Desmosome-like junctions do not occur between Sertoli cells and spermatids once the spermatids begin to elongate (Russell, 1977; Vogl et. al., 1985), implying that the desmosome-like junctions undergo turnover since they appear and disappear with particular stages of germ cell development. In addition to alterations in the Sertoli cell-germ cell junctions the cytoplasm of Sertoli cells undergoes many changes in shape during spermatogenesis, suggesting that the pattern of intermediate filaments around the nucleus, at the base of the cell, and in the apical cytoplasm may indeed change during the spermatogenic cycle.

Thesis Project

Based on the observations that: a) Sertoli cells undergo dramatic changes in cell shape during the spermatogenesis; b) Sertoli cells have a very well developed cytoskeleton; and c) actin filaments and microtubules change their
Figure 4. Electron micrographs of rat and ground squirrel Sertoli cells demonstrating IFs in different regions of the cell. a) IFs (arrowheads) at the base of the cell and around the nucleus. b) IFs in a Sertoli cell process between two developing germ cells (sp). x40,286; 1cm bar=0.25 microns. c) IFs in the immediate vicinity of desmosome-like junctions (arrows). x29,250; 1.5cm bar=0.5microns. d) Relationship between IFs around the nucleus and those at desmosome-like junctions.
Figure 5. The relationship between IFs in different areas of the Sertoli cell is unknown.
Late Spermatid

Elongate Spermatid

Spermatid

Spermatocyte

Spermatogonium

Myoid Cell

Tight Junction

?
distribution within the cell during spermatogenesis, the following questions were addressed: What is the general distribution of intermediate filaments in Sertoli cells during spermatogenesis, and how do these changes correlate with changes in the germ cell population?

No functions have specifically been attributed to intermediate filaments in Sertoli cells, although the suggestion that IFs are involved in anchorage of the nucleus within the cytoplasm would be reasonable in a system such as this where Sertoli cells change their shape while their nucleus remains in the basal portion of the cell during germ cell development. Therefore, the hypothesis that IFs are involved in the positioning of organelles within the cytoplasm was tested by attempting to perturbate the IF system. One could predict that perturbation of these filaments would result in a repositioning of organelles such as mitochondria, endoplasmic reticulum, and the nucleus. Perhaps movement of the nucleus to a more central location within the Sertoli cell could also be predicted.

Animal Models

Sprague-Dawley rats and ground squirrels (Spermophilus lateralis) were used as animal models in this study.

Because a great deal of information regarding the testis - physiology as well as morphology - has been obtained using the rat as an animal model, it seemed reasonable to study the distribution of IFs in rat Sertoli cells. Detailed staging of the spermatogenic cycle has been done in the rat (Perey et. al., 1961), allowing for correlations to be made between stages of germ cell development and IF distribution. Understanding of the involvement of cytoskeletal elements in spermatogenesis of the rat may contribute to the overall understanding of the role Sertoli cells play in this process.
Sertoli cells of the ground squirrel are large, and they contain an elaborate cytoskeleton. In addition, each Sertoli cell is associated with a small number of developing germ cells, allowing for relatively clear visualisation of spermatogenesis.
THE DISTRIBUTION OF INTERMEDIATE FILAMENTS IN SERTOLI CELLS DURING SPERMATOGENESIS

Introduction

Sertoli cells contain an elaborate cytoskeleton with each component of the cytoskeleton having a distinct distribution within the cell.

Microtubules, as observed by electron microscopy, occur in apical regions of the Sertoli cell, where they are oriented parallel to the long axis of the cell. They also occur in Sertoli cell stalks which support developing spermatids and in Sertoli cell processes that extend into the residual cytoplasm of developing spermatids. In addition, they form crescent-shaped structures in ground squirrel Sertoli cell cytoplasm adjacent to maturing spermatids (Vogl et. al., 1983; Vogl et. al., 1985). During the process of spermatogenesis the distribution of microtubules changes (Amlani & Vogl, 1986; Vogl & Amlani, 1987). Immunofluorescence data suggest that early spermatogenic cells do not have microtubules associated with them. As the round spermatids orient themselves towards the base of the Sertoli cell and begin to elongate and flatten, linear tracts of microtubules appear to surround the crypts in which these germ cells are embedded. As the spermatids undergo further maturation microtubules become concentrated adjacent to the head of the spermatid and form a C-shaped structure at this site. The C-shaped structure disappears at spermiation.

Actin filaments also change their distribution during spermatogenesis. Actin filaments, together with the plasma membrane of a Sertoli cell and a cistern of smooth endoplasmic reticulum, form ectoplasmic specializations which occur at the base of the cell adjacent to tight junctions as well as at sites adjacent to the heads of elongate spermatids. As the round spermatids
begin to elongate actin filaments surround the acrosome and a portion of the germ cell nucleus. As elongation proceeds and the spermatid heads begin to flatten, these filaments surround the entire nucleus and acrosomal cap to finally take on a fan-shaped arrangement around the flattened mature spermatid head. At spermiation, the actin filaments disappear from this region.

The general distribution of intermediate filaments in Sertoli cells has been discussed in the previous chapter. Briefly, intermediate filaments are most obvious at the base of the Sertoli cell, where they surround the nucleus. IFs have also been observed, along with microtubules, in Sertoli cell stalks which support the developing spermatids as well as in transient processes that penetrate the residual cytoplasm of the spermatid (Vogl et al., 1983; Vogl et al., 1985; Franke et al., 1979). In addition, IFs are also associated with desmosome-like junctions that occur between Sertoli cells and all stages of germ cells up to and including the round spermatid. These junctions, along with their associated filaments, do not occur adjacent to elongate spermatids, implying a reorganization of the intermediate filaments during the spermatogenic cycle.

Immunofluorescence and electron microscopy were used to investigate the possibility that changes occur in the arrangement of Sertoli cell intermediate filaments during spermatogenesis of the rat and the gold mantled ground squirrel (Spermophilus lateralis) and that changes in IF distribution correlate with changes in the germ cell population.

Antibodies are often used as a means by which to identify and localize a protein within a cell. Binding of the antibody to the protein is usually indirectly visualized, by way of a fluorescent tag bound to a secondary antibody. The latter antibody binds to epitopes on the primary antibody. Therefore, immunofluorescence allows for the localization of the protein in
question within a cell at the light microscopic level.

Electron microscopy was used to confirm that IFs were present, at the ultrastructural level, in areas of the cell corresponding to areas of fluorescence at the light microscopic level.

Preliminary reports of this study have been presented in abstract form elsewhere (Amlani & Vogl, 1986; Vogl & Amlani, 1987).

Materials and Methods

Immunofluorescence

Testes were surgically removed from rats anesthetized with sodium pentabarbitalone and were immediately embedded in Tissue Tek OCT compound (Miles Laboratories), then quick frozen in liquid nitrogen. Cryostat sections - 6 microns in thickness - were cut with a Canlab International-Harris Cryostat set at -20°C. The sections were mounted on chromic acid-cleaned glass slides and stored in moist chambers until all sections were ready for processing. The sections were permeabilized by immersion in cold acetone (maintained at -20°C with dry ice) for 10 minutes, air dried, and rehydrated with 0.1% bovine serum albumin (BSA) (Sigma) in phosphate buffered saline (PBS: 150mM NaCl, 5mM KCl, 3.2mM Na$_2$HPO$_4$, 0.8mM KH$_2$PO$_4$; pH 7.3) for 10 minutes. Slides were kept in moist chambers throughout the remainder of the experiment. The rehydrated sections were treated with 1:20 normal goat serum (NGS) in BSA buffer for 20 minutes to block any nonspecific anti-goat antigenic sites in the tissue preparation, after which they were incubated at 37°C for one hour with a commercial monoclonal mouse anti-vimentin antibody (Labsystems) diluted 1:10 with 1% NGS in BSA buffer. After three 10 minute washes with BSA buffer, the sections were incubated at 37°C for another hour.
with a goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Meloy Laboratories) diluted 1:40 with BSA buffer. After three 10 minute washes the slides were mounted with 1:1 glycerol/PBS/0.02% sodium azide and sealed with nailpolish. The slides were stored at 4°C and photographed within 48 hours with a Zeiss Photomicroscope III fitted with filters for detecting fluorescein.

The same protocol as that used for the rat was used for the ground squirrel; however, due to the delicate nature of the epithelium, the slides were coated with 0.1% polylysine (Sigma), and a Tris buffer (0.9% NaCl, 20mM Trizma Base, adjusted to pH 8.2) was used instead of PBS.

Controls consisted of the following: 1) primary antibody was omitted (control for non-specific binding of the secondary antibody), 2) both primary and secondary antibodies were omitted (control for autofluorescence), and 3) immunoblot (control for antibody specificity). For the latter, testes from six rats were removed, decapsulated and either minced for 10 minutes in 5mM EDTA-PBS or left whole. Epithelial sheets were collected from the minced tissue, washed, and resuspended in a small volume of homogenization buffer (100mM imidizole, 750mM KCl, 50mM MgCl₂, 10mM EGTA, pH 7.0). Whole testes were also transferred from 5mM EDTA-PBS to a small volume of homogenization buffer. Both preparations were homogenized for 20 strokes in a Dounce Homogenizer. A small amount of each preparation was removed for a Lowry protein assay (Lowry et. al., 1951), and the remainder was lyophilized for approximately 20 hours. The lyophilized samples were then reconstituted in standard electrophoresis sample buffer (Laemmli, 1970), boiled for 2 minutes and dialyzed overnight against sample buffer. The samples were kept frozen until polyacrylamide gel electrophoresis using sodium dodecyl sulfide (SDS) was carried out.

To further process samples for immunoblotting, SDS-polyacrylamide slab
gels (7.5%) were run according to Laemmli, 1970, using a protein concentration of 7.5 mg/ml. Molecular weight standards (Sigma Chemicals) were run alongside the testicular material. The gels were either stained with Coomasie Brilliant Blue or were transferred onto nitrocellulose paper (Biorad) using a voltage of 225V for 2 hours (modified from Towbin et. al, 1979). The nitrocellulose paper was dried, cut into strips corresponding to the lanes of the electrophoretic gel and then incubated overnight in blocking solution consisting of 5% BSA, 1.5% - 3% NGS, 0.05% Tween-20, and PBS (pH 7.3). After a brief wash, the strips were incubated at room temperature for 1 hour with monoclonal anti-vimentin antibody dilutions ranging from 1:143 to 1:167. The strips were washed again and incubated for another hour at room temperature with a goat anti-mouse IgG conjugated to horseradish peroxidase (Biorad) diluted 1:100. After another brief wash, the strips were then reacted with 4-chloro-1-naphthol (Polvino et. al., 1983) for up to 10 minutes. The peroxidase reaction was halted with distilled water.

Staging of spermatogenesis in the rat was done according to the criteria established by Perey et. al (1961). Sequencing of spermatogenesis in the ground squirrel was done using the shapes of spermatid heads and the width of the flagellum (middle piece) as indicated by Vogl & Soucy (1985).

Electron Microscopy

Testes were surgically removed from rat and ground squirrel. The testicular artery was cannulated with a 25G needle and the testes were perfusion-fixed with 1.5% paraformaldehyde/1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (adjusted to pH 7.3) for 30 minutes. The testes were then cut into small cubes and immersion-fixed in the same fixative for 1.5 hours. The tissue was then washed three times - 10 minutes per wash - with 0.1 M
sodium cacodylate, post-fixed in 1% osmium tetroxide in sodium cacodylate for 1 hour on ice. After 3 - 10 minute washes with distilled water, the tissue was stained en bloc with 1% aqueous uranyl acetate for 1 hour, washed 3 times, and then dehydrated with series of ethanol (50%, 75%, 95%, 2x100%), followed by two treatments of propylene oxide. The tissue was left overnight in 50% propylene oxide/50% epon, embedded in 100% epon the following day and cured for approximately 36 hours. Thick sections - 1 micron in thickness - were cut using glass knives. The sections were stained with Toluidine Blue, and then photographed using a Zeiss photomicroscope III. Thin sections (600nm) were cut using a diamond knife (Diatome). The sections were stained with 1% uranyl acetate in methanol and Reynold's lead citrate, and then were viewed and photographed with a Phillips 300 transmission electron microscope.

Results

Sertoli cells and spermatogenic cells within cross-sections of seminiferous tubules were identified with phase optics by cell shape and cell association. Rough staging of spermatogenic cells - that is, early, intermediate, or late - was accomplished by examining the position of elongate spermatids within the epithelium and by examining the shape of spermatid heads. Specific stages of spermatogenesis in the rat were assigned according to the stages of Perey et. al. (1961).

Fluorescence within Sertoli cells occurred at the base of the cell and around the nucleus at all stages of germ cell development, although changes in the fluorescence pattern occurred in the apical cytoplasm.

At early stages of spermatogenesis in the rat, where elongating spermatids are embedded in Sertoli cell crypts deep within the seminiferous epithelium...
(stages II - IV), fluorescence was emitted at the base of the Sertoli cell, around the nucleus, and in regions of Sertoli cell crypts containing elongating spermatids (Figure 6a,a',b,b'). As the spermatids develop and move upwards within the epithelium (stages V - VI), fluorescence in the area of the crypts diminished and was evident only at the base of the Sertoli cell (Figure 6c,c',d,d'). Once the elongate spermatids are at the apex of the seminiferous epithelium, ready for release into the lumen (stages VII - VIII), fluorescence was emitted only at the base of the cell (Figure 6e,e'). No fluorescence was evident in the regions of the Sertoli cell close to the elongate spermatids. Linear tracts of fluorescence reappeared in the cytoplasm of Sertoli cells amongst the next generation of elongating spermatids (stages XIV) (Figure 6f,f').

The pattern of fluorescence in ground squirrel Sertoli cells was similar to that in the rat; that is, fluorescence was emitted at the base of the Sertoli cell and in the area of the crypts housing developing spermatids. Figure 7 illustrates a few stages of ground squirrel spermatogenesis. Changes in the pattern of fluorescence became less intense in crypt areas as the spermatids continued to elongate. At a later stage of germ cell development, fluorescence was brightest at the base of the Sertoli cell; however, unlike in the rat, a complex network of fluorescent strands could be seen throughout the Sertoli cell (Figure 7d,d'). Also, bright patches of fluorescence were observed immediately adjacent to the head of the maturing spermatids.

Desmosome-like junctions were not detected with fluorescence because few IFs are associated with a desmosome plaque, and IFs only associate with the plaque on the Sertoli cell side of the junction. In addition, desmosome-like junctions are often located in close proximity to the nucleus of Sertoli cells, making it difficult to distinguish perinuclear IFs from those
Figure 6. A series of fluorescence and phase micrographs illustrating the changes in anti-vimentin staining of rat Sertoli cells during spermatogenesis. (a,a',b,b') show early stages of spermatogenesis where fluorescence is evident around Sertoli cell nuclei (arrows) and in regions around Sertoli cell crypts (arrowheads) containing elongate spermatids. (c,c',d,d') are at a later stage of spermatid development, where fluorescence is reduced in apical regions of Sertoli cells and occurs mainly around Sertoli cell nuclei. (e,e') illustrate mature spermatids near the apex of Sertoli cells (open arrows). Note that fluorescence occurs only at the base of the Sertoli cell. (f,f') shows linear tracts of fluorescence in Sertoli cell cytoplasm amongst the next generation of elongating spermatids (straight arrows). x996; 0.99cm=1micron.
Figure 7. Shown here are fluorescence and phase micrographs illustrating anti-vimentin staining in ground squirrel Sertoli cells during spermatogenesis. Arrows indicate Sertoli cell nuclei. Arrowheads indicate heads of spermatids. x933; 0.93cm=1 microns.
associated with desmosome-like junctions.

The specificity of the anti-vimentin antibody was tested by immunoblotting. A standard SDS-polyacrylamide gel electrophoresis of homogenized testes — both whole testes and epithelial sheets — revealed several polypeptide bands when stained with Coomassie Brilliant Blue, including a band at 58,000 molecular weight (MW), which corresponds to the molecular weight of vimentin (Fig. 8, lanes 2 & 3). Molecular weights of the polypeptides were determined according to molecular weight standards (Fig. 8, lane 1). Immunoblots (Fig. 8, lanes 4 & 6) revealed one dark staining band corresponding to 58,000 MW. Immunoblots of the whole testes homogenate also showed faint bands at 97,000 MW and 61,000 MW, as well as a very faint band at 55,000 MW. The latter faint band also appeared on immunoblots of epithelial sheet homogenates. The staining at 97,000 MW was confirmed as non-specific by its presence on immunoblots of whole testes homogenates treated only with secondary antibody (Fig. 8, lane 8). The other bands — at 61,000 MW and 55,000 MW — cannot be ruled out as being non-specific since they did not appear on the control immunoblots. Therefore, the specificity of the antibody is not absolute. Immunoblots of epithelial sheet homogenate that were treated only with secondary antibody (Fig. 8, lane 10) showed no non-specific staining. Because staining at 55,000 MW appears on immunoblots of both whole testes and epithelial sheets, the polypeptide may be a proteolytic product of the 58,000 MW vimentin protein. Finally, to ensure the transfer of the polypeptides from the electrophoretic gels to the nitrocellulose paper, all immunoblots were post-stained with Amido Black (Fig. 8, lanes 5,7,9,11).

Controls for specificity of the secondary antibody used in the immunofluorescence studies showed no fluorescent staining in the tissue sections when the primary antibody was omitted (Fig. 9b,b'). Likewise,
Figure 8. Immunoblots of whole testis and isolated seminiferous epithelium. Lane 1: SDS-PAGE of molecular weight standards. Lanes 2 & 3: SDS-PAGE of isolated epithelial sheets and of whole testes, respectively. Lanes 4 & 6: Immunoblots of whole testis and epithelial sheets, respectively. Note the strong anti-vimentin antibody reaction with a band at approximately 58,000 daltons in each blot. Lanes 8 & 10: Control immunoblots in which the primary antibody was ommitted. Notice the weak non-specific binding (arrowhead) in lane 8 that corresponds to a similar binding in lane 4. Lanes 5, 7, 9 & 11: Imido black stained nitrocellulose strips after immunoblotting (lanes 4, 6, 8, & 10, respectively). These results confirm the transfer of polypeptides from the SDS-PAGE gels to the nitrocellulose strips.
Figure 9. Shown here are controls for the localization of vimentin by immunofluorescence. (a,a') Primary and secondary antibodies. (b,b') Secondary antibody only. (c,c') Both primary and secondary antibody omitted. x1184; 1.18cm=1 microns.
controls for autofluorescence of the tissue sections showed no fluorescent staining (Fig. 9c, c'). An example of specific fluorescence staining - along with the corresponding phase micrograph - is illustrated in panel a, a' of Fig. 9.

Electron microscopy confirmed the presence of intermediate filaments in areas of the Sertoli cell indicated by fluorescence in both the rat and ground squirrel. IFs were always present at the base of Sertoli cells, and around the nuclei at all stages of germ cell development. At the stage of spermatogenesis in the rat, where spermatids are embedded in Sertoli cell crypts deep within the seminiferous epithelium. IFs were consistently observed adjacent to the convex aspect of spermatid heads (Figs. 10 & 11). Cross sectional profiles of these filaments occurred in groups of approximately eight filaments. They were identified as intermediate filaments based on their diameter and on the fact that they were intermediate in size between microtubules, found in Sertoli cell cytoplasm adjacent to spermatid heads, and actin filaments of the ectoplasmic specializations adjacent to spermatid heads. The intermediate filaments appeared to be in close association with the endoplasmic reticulum and also with the actin filaments of ectoplasmic specializations (Figs 10 & 11). Intermediate filaments could not be located adjacent to spermatid heads at later stages of germ cell development, where spermatids were in crypts at the apex of the epithelium (Fig. 12). Figure 13 illustrates intermediate filaments in Sertoli cell stalks supporting elongate spermatids of the ground squirrel. The filaments in this area in situated close to the heads of the spermatids.
Figure 10. An electron micrograph illustrating Sertoli cell crypts at an early stage of spermatogenesis. Note the group of intermediate filaments (arrowheads), in cross-sectional profile, present at the convex aspect of the spermatid heads. x 48,335; 1.2cm = 0.25 microns.
Figure 11. A series of electron micrographs illustrating Sertoli cell crypts at an early stage of spermatogenesis. Note the group of intermediate filaments consistently present at the convex aspect of the spermatid heads. a: x 38,607, 1.0cm = 0.25microns; b, c: x 63,462, 1.6cm = 0.25microns; d: x 48469, 1.2cm = 0.25microns; e: x 60,000, 1.5cm = 0.25microns.
Figure 12. Electron micrographs demonstrating a lack of IFs in rat Sertoli cell crypts adjacent to heads of mature spermatids. The stage of germ cell development corresponds to that illustrated in panel e,e' of figure 6.
Figure 13. Electron micrographs demonstrating IFs in ground squirrel Sertoli cell stalks supporting elongate spermatids (sp). IFs observed in this area correspond to areas of fluorescence adjacent to spermatid heads in panels a,a' and b,b' of figure 7. x29,375; 1.5cm=0.5 microns.
DISCUSSION

The results of the present study are consistent with the general distribution of intermediate filaments in mammalian Sertoli cells described previously by Fawcett (1975), Vogl et. al. (1983), Vogl et. al. (1985), Russell (1977), and Franke et. al. (1979). They further demonstrate that the distribution of intermediate filaments changes during spermatogenesis. Both immunofluorescence and electron microscopy illustrate that intermediate filaments are consistently located in the basal portion of the cell. Immunofluorescent staining of IFs with anti-vimentin antibody confirms the results of Franke et. al. (1979) that IFs in Sertoli cells are composed of vimentin. The results also demonstrate a specific association of IFs with ectoplasmic specializations surrounding elongate spermatids.

Intermediate filaments are observed around the Sertoli cell nucleus at all stages of germ cell development, with tracts of vimentin filaments extending from the nucleus up to Sertoli cell crypts during stages where elongating spermatids are embedded in Sertoli cell crypts deep within the seminiferous epithelium. In the rat, vimentin filaments disappear from the crypt areas, and essentially from apical regions of the cell, when mature spermatids reach the apex of the epithelium and are ready to be released. The reappearance of vimentin in and amongst the crypts at the time that the next generation of round spermatids begins to elongate suggests that IFs may be involved in supporting and maintaining the crypts in a perinuclear position during the early and intermediate stages of spermatogenesis.

It is interesting that IFs in Sertoli cell crypts are consistently and specifically associated with the convex (dorsal) aspect of spermatid heads at a specific stage of the spermatogenic cycle, and then disappear from this
region when the spermatids are no longer embedded deep within the seminiferous epithelium but are residing in shallow crypts near the apex of the epithelium. Perhaps IFs are involved in anchoring Sertoli cell crypts deep within the epithelium at appropriate stages of the spermatogenic cycle and disappear from this location when it is no longer necessary for spermatids to be deeply embedded within the seminiferous epithelium.

In ground squirrel Sertoli cells vimentin filaments change their distribution somewhat during spermatogenesis, although they do not disappear from the apical cytoplasm. They remain as a fine network of filaments throughout the cell. Elongate spermatids in this animal are not pulled down deep into the epithelium at any stage, implying that their involvement in the crypt areas may not be in supporting and maintaining the crypts, but may be in lending structural support to the Sertoli cell stalks which support mature spermatids. In the ground squirrel IFs may be involved in maintaining the general architecture of Sertoli cells.

Although specific functions for IFs in Sertoli cells have not been determined, several functions have been suggested for IFs in general. Maintenance of cell shape and anchorage of cytoplasmic organelles and the nucleus are two of the more common roles attributed to this component of the cytoskeleton. In Sertoli cells, the appearance of vimentin in and amongst those crypt areas present only at certain stages of germ cell development in the rat is consistent with the hypothesis that IFs somehow may be involved in intercellular attachment and therefore in organizing the seminiferous epithelium. Also, the constant location of vimentin around Sertoli cell nuclei is consistent with the idea that intermediate filaments position the nucleus within the cell.

Sertoli cells are dynamic cells which continually undergo morphological as
well as biochemical and physiological changes. Alterations in Sertoli cell shape may be due to rearrangements of the cytoskeleton. It has been reported that responsiveness of Sertoli cells to FSH changes during the spermatogenic cycle (Parvinen et al., 1980), and that vimentin filaments in these cells are phosphorylated in a FSH-dependent manner (Spruill et al., 1983a) as well as in a calcium/calmodulin-dependent and cAMP-dependent and -independent manner (Spruill et al., 1983b). Perhaps phosphorylation of vimentin filaments in response to changes in FSH levels or changes in calcium levels, or in response to activation of cAMP-dependent pathways induces changes in the organization of these filaments, and may be partially responsible for the shape changes undergone by Sertoli cells during the process of spermatogenesis. As stated previously, intermediate filaments are found in close association to ectoplasmic specializations at particular stages of spermatogenesis. Ectoplasmic specializations are partially composed of endoplasmic reticulum, which may, as in other cell types, be involved in storing and/or sequestering calcium in response to some stimulus. It is tempting to speculate that intermediate filaments, phosphorylated in response to altered calcium levels, may reorganize their distribution and serve to strengthen the sites of adhesion between Sertoli cells and spermatids when the spermatids are embedded deep within Sertoli cell crypts.

In summary, the data demonstrate that vimentin intermediate filaments in Sertoli cells change their distribution during the spermatogenic cycle, as do actin filaments (Vogl & Soucy, 1985) and microtubules (Amlani & Vogl, 1986; Vogl & Amlani, 1987; Amlani & Vogl, 1987). Changes in the organization of all three cytoskeletal components within a cell which undergoes dramatic alterations in cell shape strongly suggest that the cytoskeleton in Sertoli cells plays a major role in these shape changes.
INTRODUCTION

It has previously been established that actin filaments and microtubules change their distribution in Sertoli cells during the spermatogenic cycle (Vogl & Soucy, 1985; Vogl et. al., 1986; Vogl et. al., 1983, Amlani & Vogl, 1986; Vogl & Amlani, 1987). Immunofluorescent localization of vimentin filaments in Sertoli cells, confirmed by electron microscopy, establishes that the distribution of this component of the cytoskeleton also changes during spermatogenesis. The location of vimentin filaments at the base of the Sertoli cell, around the nucleus, in Sertoli cell stalks and processes, and at desmosome-like junctions, as well as their transient appearance in Sertoli cell crypts at particular stages of germ cell development suggests that they may be involved in: a) maintaining cell shape; b) supporting Sertoli cell crypts when the crypts are located deep within the seminiferous epithelium, and c) anchoring the nucleus to the base of the Sertoli cell.

The next step in the investigation of intermediate filaments in Sertoli cells is to experimentally test the hypotheses relating to function. A method commonly used in the experimental testing of an hypothesis regarding function is to perturbate the component in question and study the consequences. The function attributed to that component of a cell should no longer occur or should be altered in some way if the hypothesis is true. Perturbation of microtubules is one example of this approach and has been carried out in several cell types including Sertoli cells (Russell et. al.,
Treatment of rat and squirrel testes with colchicine and vinblastine indicate that Mts are generally involved in maintaining the columnar shape of the Sertoli cell. Treatment with these agents results in a shorter, more rounded Sertoli cell in which stalks, which normally support elongate spermatids, do not form.

A specific perturbant of intermediate filaments has not been available until very recently. Attempts have been made to disrupt the IF system by microinjecting monoclonal antibodies into a cell. Microinjection of antibodies against keratins and vimentin (Eckert & Daley, 1981; Eckert et al., 1984; Gawlitta et al., 1981 Klymkowsky, 1982; Lane & Klymkowsky, 1984) have succeeded in disrupting IFs although no significant changes in cell shape and motility are observed. Lane & Klymkowsky (1984) proposed that no significant effects were observed upon the disruption of IFs because the cells were not adequately stressed to determine the function of IFs.

Microtubule inhibitors such as taxol and nocodazole have also been utilized to indirectly study IFs and their relationship with Mts (Geuens et al., 1983; Green & Goldman, 1983). Both groups found rearrangement of IFs as a secondary event to microtubules rearrangement. Disruption and rearrangement of more than one cytoskeletal component makes clear interpretation of data with respect to the function of each component difficult.

Recent reports (Durham et al., 1983; Eckert, 1985 and 1986) indicate that intermediate filaments can specifically be disrupted in cultured cells with low concentrations of acrylamide (5mM), resulting in the aggregation of IFs around the nucleus. Acrylamide is a neurotoxin which appears to affect the nervous system of mammals. It causes neuronal axonopathy, characterized by the accumulation of neurofilaments within the terminal ends of axons in both the central and peripheral nervous systems (Cavanaugh & Gysbers, 1983; Howland
& Ali, 1986; Le Quesne, 1985; Spencer & Schaumberg, 1977a,b). Acrylamide is a vinyl monomer used in industry (Tilson, 1981) as a flocculant in the treatment and clarification of effluents and in the production of ores and coals, as a grouting agent, and as a strengthener of paper and cardboard. As a polymer acrylamide is used in the disposal of industrial wastes and in water purification as an algacide and bactericide. Polyacrylamide is commonly found in adhesives and photographic supplies, and is also used as a coating for metals. In research, polyacrylamide gels are used as a medium with which to separate proteins on the basis of molecular weight.

The association of neuronal disorders with acrylamide exposure has been known for several years (Spencer & Schaumberg, 1970), although the mechanism by which acrylamide has its effect is yet undetermined. Many investigators (Srivastava et. al., 1985; Sakamoto & Hashimoto, 1985a,b; Hashimoto & Ando, 1973; Hashimoto & Aldridge, 1970; Howland, 1985; Brimijoin & Hammond, 1985; Tanii & Hashimoto, 1981; Miller et. al., 1982) have shown that acrylamide affects certain metabolic pathways involving glycolytic enzymes — specifically enolases and glyceraldehyde-3-phosphate dehydrogenase — and also affects amino acid incorporation into proteins.

Effects of acrylamide on hormonal levels and on mating processes have been studied in the rat. Ali et. al. (1983) reported that repeated injection of acrylamide results in decreased testosterone levels in the blood, implying that testicular function may be affected. In 1986, Zenick & Hope reported that copulatory behavior, ejaculatory processes, and transport of sperm to the uterus are disrupted after repeated administration of acrylamide. These studies imply that the testes are susceptible to the effects of acrylamide intoxication.

At the ultrastructural level, treatment with acrylamide results in the
accumulation of various organelles at the distal ends of axons in both the central and peripheral nervous systems. In cultured fibroblasts and in PtK2 cells respectively (Durham et. al., 1983; Eckert, 1985 & 1986) IFs aggregate near the nucleus, while other cytoskeletal components remain unaffected. With time lapse video techniques, Eckert (1985, 1986) observed that mitochondria also collapsed around the nucleus along with the IFs, and showed that although the mitochondria were still able to move to peripheral areas of the cell—presumably along tracts of microtubules (Allen et. al., 1985; Vale, R.D. et. al., 1985a,b; Miller et. al., 1985)—they were unable to remain in these areas and consistently collapsed around the nucleus. Nuclei within these cells continually spun. In addition, cell contact could no longer be established.

Based on the reports that acrylamide appears to disrupt the IF system without causing disruption of other cytoskeletal elements and without causing toxic metabolic effects at low concentrations, the hypothesis that intermediate filaments may serve to anchor the nucleus to the base of the Sertoli cell could be tested by using acrylamide as a perturbant of vimentin filaments in Sertoli cells. The method chosen to expose Sertoli cells to acrylamide was to perfuse the testes with the perturbant. Hoffer et. al. (1983) reported that perfusion of testes is a valid method for studying the effects of drugs and studying hormone metabolism. One advantage to using isolated organs is that they maintain the overall structure and intercellular relations of the organ in vivo. Metabolic pathways can be maintained by providing the organ with oxygen, ions, and an energy source and does not appear to cause damage to the tissue at the ultrastructural level.
MATERIALS AND METHODS

Testes were surgically removed from seven rats and four ground squirrels. The testicular artery was cannulated using a 25 gauge needle and were perfused with a standard rat Krebs-Ringer Bicarbonate buffer (Palaty, 1971) containing 4% dialyzed bovine serum albumin (Fraction V, Sigma Chemicals) and 5mM, 10mM, 50mM and 100mM acrylamide. The duration of perfusion ranged from 2 hours up to 8 hours. The perfusate was warmed to 33°C with a water bath, and 5% CO₂/95% O₂ was continually bubbled into the solution. The perfusion apparatus was modified from that of Brunengraber et. al. (1975) and Hoffer et. al. (1983). A schematic diagram of the apparatus is shown in figure 14. After a designated period of exposure to the experimental solution, the perfusate was switched via a three-way stopcock to fixative for electron microscopy (1.5% glutaraldehyde, 1.5% paraformaldehyde, 0.1M sodium cacodylate, adjusted to pH 7.3). Testes were perfused with fixative for 30 minutes, after which the tissue was cut into 1mm cubes and immersed in the fixative for 1.5 hours. The tissue was then processed for electron microscopy according to standard procedures described in Chapter 2.

RESULTS

Treatment of rat and squirrel testes with a concentration of 5mM acrylamide for a period of two hours yielded no observable effects on Sertoli cells, both at the light and electron microscopic level. Toluidine blue
Figure 14. Schematic diagram of the perfusion apparatus utilized in IF perturbation experiments. The perfusate, bubbled with 5% CO$_2$/95%O$_2$, was warmed to 33°C and gravity fed into the testicular artery of rat and ground squirrel testes.
stained one micron thick sections of treated rat testes (Fig. 15a) show no adverse effects on the seminiferous tubules or the interstitial tissue. The seminiferous epithelium is relatively intact, and is virtually identical to that in control sections (Fig. 15b). The Sertoli cells maintain their intimate relationship with the developing germ cells and also maintain their cell shape. Contrary to what was anticipated, the nuclei of Sertoli cells remained at the base of the cell.

At the ultrastructural level (Fig. 16) Sertoli cell nuclei in treated tissue are no different from those in controls with respect to their location and shape. Intermediate filaments do not appear to have been affected by acrylamide; their distribution around Sertoli cell nuclei within Sertoli cell stalks and in association with desmosome-like junctions is maintained and does not differ from that within Sertoli cells in control samples.

To exclude the possibility that acrylamide was not in a high enough concentration to diffuse through the interstitial tissue and peritubular tissue to the Sertoli cells, its concentration was increased to 10mM and the duration of perfusion was lengthened from an initial 2 hours to 4 hours, and eventually to 8 hours. Figures 17a,b and 18 illustrate that no effect was seen at either the light or electron microscopic level. The tissue is again relatively intact; the seminiferous epithelium does not show any signs of damage or toxicity, nor does it show a shift in the position of Sertoli cell nuclei. The general distribution of IFs within Sertoli cells is once again maintained.

Higher concentrations of 50mM and 100mM acrylamide were perfused through the testes of both rats and ground squirrels. At these concentrations nuclear material of the developing spermatocytes and round spermatids exhibit a slightly different pattern from that normally seen (Figs. 17c,d & 20). Round spermatids
separated from Sertoli cells in tissue exposed to 100mM acrylamide (Fig. 20); however this may simply be due to osmotic effects of the acrylamide since these effects were not controlled for. Acrosome formation appears to have been affected in that the acrosomal membrane appears "wavy" at the light microscopic level (Fig. 20a) and is vesciculated at the electron microscopic level (Fig. 22a). Figure 22b illustrates an area of accumulation of mitochondria within Sertoli cell cytoplasm as well as within that of developing germ cells. Quantitative analysis of this observation was not carried out. Figures 19 and 21 demonstrate IFs not only at the base of the Sertoli cell but also within apical portions of these cells as well as in association with desmosome-like junctions. The position of Sertoli cell nuclei remained basal, and the IF system was still unaffected.
Figure 15. Light micrographs of 1 micron toluidine blue-stained thick sections of seminiferous epithelium after treatment with 5mM acrylamide for 2 hours. No difference in overall morphology or location of Sertoli cell nuclei (arrows) can be seen between experimental (a) and control samples (b). bv=blood vessel. x640; 1.28cm=2 microns.
Figure 16. A series of electron micrographs illustrating IFs (arrowheads) in Sertoli cells after treatment with 5mM acrylamide for 2 hours. Panels a and b demonstrate IFs (arrowheads) around a Sertoli cell nucleus (Nuc), and in a Sertoli cell stalk. IFs in the latter panel are also associated with desmosome-like junctions (arrows). Panels c and d demonstrate similar regions in control tissue. a & c: x27,500, 1.38cm=0.5 microns; b & d: x20,563, 1cm=0.5 microns.
Figure 17. Light micrographs of 1 micron toluidine blue-stained thick sections of seminiferous tubules after treatment with different doses of acrylamide. a and c show seminiferous epithelium after treatment with 10mM and 50mM acrylamide respectively, while b and d show corresponding regions in control tissue. No difference is seen in nuclear position (arrows) and overall morphology. ×1315; 1.3cm=1 microns.
Figure 18. A series of electron micrographs illustrating Sertoli cell regions after treatment with 10mM acrylamide for 4 hours (a,b,c) and 8 hours (d,e). No difference in tissue morphology can be seen with different perfusion times. Nuc= Sertoli cell nucleus; sp=spermatid; arrowheads=IFs; arrows=desmosome-like junctions. a,b,c,e: x19583, 1.0cm=0.5 microns; d: x17140, 0.85=0.5 microns.
Figure 19. Electron micrographs illustrating Sertoli cell regions after treatment with 50mM acrylamide. No difference in IF distribution can be seen between treated tissue (a,b) and control (c,d). Arrowheads=IFs. a,b: x13,950, 1.4cm=1 microns; c,d: x19583, 1.95=1 microns.
Figure 20. Light micrographs of 1micron toluidine blue-stained thick sections of seminiferous tubules after treatment with 100mM acrylamide for 8 hours (a). Although there is no change in nuclear position (Nuc), possible toxic effects are apparent. Round spermatids appear to have pulled away from Sertoli cells (arrows). Panel b shows a corresponding seminiferous tubule in control tissue. x606; 1.2cm=2 microns.
Figure 21. Electron micrographs of Sertoli cells treated with 100mM acrylamide (a,b). Panels a and c demonstrate IFs (arrowheads) around the nucleus. Panel b demonstrates IFs in a Sertoli cell stalk. Panel d demonstrates IFs in Sertoli cell cytoplasm above the nucleus. x20460; 1.0cm= 0.5 microns.
Figure 22. Electron micrographs demonstrating possible toxic effects within the seminiferous epithelium after perfusion with 100mM acrylamide. a) Vesiculation of the acrosomal membrane of an elongating spermatid (outlined arrowheads). IFs (arrowheads) are present in their normal arrangement within the adjacent Sertoli cell stalk. b) Accumulation of mitochondria within the cytoplasm of developing germ cells (M) as well as in that of Sertoli cells (m). a: x5,134; 1.0cm=2 microns; b: x13,419, 1.3cm=1 microns.
DISCUSSION

The results presented in this study demonstrate that acrylamide, a disruptor of intermediate filaments, has no effect on the distribution of vimentin filaments in Sertoli cells in vivo when rat and ground squirrel testes were perfused with acrylamide at concentrations of 5mM to 100mM. The data obtained from these studies are contrary to those of Eckert (1985, 1986) and Durham et. al. (1983). Both laboratories demonstrate that upon exposure of cultured PtK1 cells and fibroblasts to low concentrations of acrylamide intermediate filaments within these cells no longer formed a continuous network which normally extends from the nucleus to the periphery of the cell, but collapsed around the nucleus. Cytoplasmic organelles such as mitochondria and endoplasmic reticulum also accumulated around the nuclear region.

The results documented in this study are consistent with reports that acrylamide has no effect on fibroblasts in vivo (Durham et. al., 1983) and that some of the cytotoxic effects of acrylamide treatment can be prevented in vitro upon the addition of metabolic coenzymes such as NAD, NADP, nicotinamide and glutathione (Sharma & Obersteiner, 1977), which would be present in an in vivo situation. These investigators suggest that an acrylamide effect is observed in vitro because certain metabolic pathways in cultured cells are non-functional, thus allowing for the accumulation of acrylamide within these cells over time.

An obvious difference between cells treated with acrylamide in vitro and those treated in vivo is that the perturbant is in direct contact with cells in the former situation, whereas cells in vivo are not directly exposed to the perturbant. Under in vivo conditions acrylamide must make its way through the vascular endothelium to the interstitial tissue, and then to the seminiferous
tubules. Upon reaching the tubules acrylamide must make its way through the peritubular tissue to the base of the Sertoli cells. Miller et. al. (1982) claim that acrylamide is a highly polar compound and its absorption into organs such as liver, kidney and testes would be delayed because of the high lipid content within these organs. In addition, Dixit et. al. (1986) recently reported that blood serum proteins have a relatively high affinity for acrylamide. The buffer used in this study contained the blood serum protein albumin. Its binding of acrylamide may account for the lack of observable effects in tissue perfused with low concentrations of acrylamide.

In light of these reports, higher concentrations of acrylamide were given for longer periods of time in order to allow the acrylamide to overcome the various barriers already mentioned. There is little doubt that acrylamide was indeed reaching Sertoli cells since toxic effects such as vesiculation of acrosomal membranes, aggregation of mitochondria, and alteration in chromatin pattern were observed. Shiraishi (1978) reported that repeated administration of acrylamide induces chromatid exchange and chromatid breaks in spermatogonia in mice. His observations provide some support for toxic effects observed in this study in that abnormal chromatin patterns may be a result of acrylamide intoxication.

It appears that the effects of acrylamide intoxication are mostly observed after repeated administration of the chemical as opposed to a single dose. For the purposes of this study, repeated administration would not have been beneficial. Firstly, repeated administration of acrylamide to the animals, presumably via their diet, would have resulted in the clinical condition of neuronal axonopathy as well as changes in neuropeptide and reproductive hormone levels (Ali et. al., 1983; Zenick & Hope, 1986). All these changes would introduce numerous variables, making interpretation of the data
difficult. In addition, for ethical reasons the alternative methods of inducing acrylamide effects were utilized.

Although effects of acrylamide on metabolic pathways and on intermediate filaments have been documented, and clinical cases suggest that its effects are cumulative, its mode of action is yet to determined. Recent reports suggest that acrylamide does not in fact enter the cell, but appears to initiate its effects at the cell membrane. It is plausible that acrylamide binds in some manner to the plasma membrane, setting off a kinase cascade reaction, which in turn results in an alteration of phosphorylation pathways. By affecting these pathways it may have its effects not only on metabolic pathways but also on intermediate filaments. Howland & Alli (1986) report an alteration in the degree of phosphorylation of neurofilament proteins upon treatment of spinal cord preparations with acrylamide. Also, NMR techniques reveal that keratin filament proteins were dephosphorylated in PtK1 cells treated with acrylamide (Eckert, 1986).

It becomes tempting to speculate that the cumulative effects of acrylamide poisoning observed in industry may be due to an alteration of phosphorylation process with a cell, thus altering metabolic pathways and intermediate filament organization and resulting in a collapse of IFs either at distal ends of axons or around the nucleus, depending on the cell type.

Before acrylamide can be used conclusively to test hypothesis in vivo its mode of action requires further investigation. Although no conclusions regarding intermediate filament function in Sertoli cells can be made based on this study, the lack of an acrylamide effect on intermediate filaments within Sertoli cells serves to emphasize the degree to which intermediate filaments form a strong and stable network within a cell.
SUMMARY AND FUTURE CONSIDERATIONS

The studies detailed in this thesis were undertaken first of all to determine whether intermediate filaments in Sertoli cells, composed of vimentin, change their distribution during the spermatogenic cycle of the rat and the ground squirrel *S. lateralis*, and if so, to determine whether these changes correlate with ongoing changes in the germ cell population. Secondly, perturbation experiments were carried out in order to test the hypothesis that intermediate filaments play a role in anchoring the nucleus within the cytoplasm of the cell.

The following conclusions regarding intermediate filaments can be drawn from the results of the project:

1) The distribution of vimentin filaments in rat Sertoli cells changes during the spermatogenic cycle.

2) The general distribution of vimentin filaments in ground squirrel Sertoli cells are consistent with those in the rat, although changes in their distribution during spermatogenesis are not as dramatic. Vimentin filaments in these cells may only be structural in these cells and may not serve to strengthen sites of attachment between Sertoli cells and spermatids.

3) Intermediate filaments in the rat are closely associated with those Sertoli cell crypts deep within the seminiferous epithelium in which elongate spermatids at certain stages of sperm development are embedded. The filaments are morphologically
related to ectoplasmic specializations of these crypts and may be involved in attachment and positioning of germ cells within the seminiferous epithelium.

4) The intermediate filament perturbant acrylamide has no obvious effect on the apparent distribution of intermediate filaments in Sertoli cells in vivo.

No conclusions can be made regarding the hypothesis that intermediate filaments serve to anchor the nucleus within the cytoplasm of Sertoli cells because the intermediate filaments were not disrupted upon perfusion of acrylamide into the testis. Perhaps a more direct application of the perturbant on Sertoli cells, such as incubation of cultured Sertoli cells in medium containing acrylamide, may yield more obvious effects.

Considerations for future studies on intermediate filaments in Sertoli cells include further investigation of the filaments associated with Sertoli cell crypts at particular stages of spermatogenesis. The spermatogenic cycle is under hormonal regulation as are Sertoli cells. Vimentin filaments are responsive to FSH in that they can be phosphorylated in a FSH-dependent manner. Therefore, it would be valuable to determine whether intermediate filaments are phosphorylated at particular stages of spermatogenesis. These studies would perhaps shed some light on factors affecting intermediate filament distribution within Sertoli cells. Other equally important studies regarding intermediate filaments in crypt areas include determining the relationship that intermediate filaments have with ectoplasmic specializations located at junctions between Sertoli cell crypts and elongate spermatids.
residing in these crypts. Do these filaments penetrate ectoplasmic specializations, and if so, what is their relationship to the actin filaments and smooth endoplasmic reticulum which make up part of the ectoplasmic specializations?

In addition to studying intermediate filaments in crypt areas, it would be interesting to study, in more detail, intermediate filaments associated with desmosome-like junctions. It would be important first of all, to characterize these junctions; that is, to identify their components and determine whether these components are similar to those of the classical desmosomal junction. It would be important to demonstrate conclusively - using immunologic techniques - that at the ultrastructural level intermediate filaments associated with these junctional sites are composed of vimentin, and that this class of intermediate filaments binds either to desmoplakins in a similar fashion to intermediate filaments composed of keratins, or to other elements unique to desmosome-like junctions in Sertoli cells.

Demonstration of changes in intermediate filament distribution in Sertoli cells during spermatogenesis and of their close association with Sertoli cell crypts at specific stages of germ cell development forms a foundation upon which specific functions of vimentin intermediate filaments in Sertoli cells can be based.
REFERENCES


Capco, D.G., Krochmalnic, G. & Penman, S. A new method of preparing


Sauk, J.J., Krumveide, M., Cocking-Johnson, D. & White, J.G. Reconstitution of cytokeratin filaments in vitro: further evidence for the role of


Sun, T-T & Green, H. Immunofluorescent staining of keratin fibres in cultured cells. Cell. 1978, 14: 469 - 676.


