COMPARATIVE STUDIES ON GONAD DEVELOPMENT IN EUTHERIAN AND MARSUPIAL MAMMALS

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by

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To

My husband, Saleh,

and to my family

Declaration

I hereby declare that this thesis is my own composition, and that, except, where otherwise stated, the experimental work was performed by me alone.

None of the material in this thesis has been submitted for any other degree.

Mahin Dairi

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ABSTRACT

The origins of the somatic cells of the mammalian gonad are still controversial. This histological study was prompted by a lack of information on this topic in marsupials. An attempt has been made to trace the origins of the blastema, granulosa cells, polyovular follicles (POFs), interstitial tissue (IT) and the rete ovarii.

This study provides a description of gonad development in mouse (early stages only) and 3 macropod species, namely: the tammar wallaby, the bettong and the potoroo.

It was found that the blastema in both the mouse and the macropods has a dual origin. While in mouse embryos it arises from the mesothelial and mesonephric tubule cells, in the macropods studied it derives from the mesothelium and the mesenchyme.

In contradistinction to some eutherians, in the bettong and potoroo the granulosa cells derive from the medullary cords which have a blastemal origin.

The ovaries of the mouse, bettong and potoroo are similar in that they belong to the type with immediate meiosis. Histochemical and ultrastructural work supports this conclusion: characteristically HSD enzyme was absent from potoroo and Bennett's wallaby ovaries at early stages of development.

In the potoroo POFs arise from isolated oocytes becoming surrounded by a common envelope of granulosa cells, while the IT derives from the medullary cords.

The mode of development of the rete in the potoroo is similar to that reported for some eutherians and all marsupials investigated: it forms from a condensation of cells at the anterior end of the gonadal rudiment. The extent to which it develops within the ovaries, however, varies in different marsupials.

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

1.1. OBJECTIVES

The general aims of this study are as follows:

1) To compare the process of gonadal sexual differentiation and oogenesis in eutherian and marsupial mammals.

2) To tackle a number of specific and still unresolved problems in mammalian gonadogenesis.

To achieve the first objective the mouse has been chosen as the representative eutherian and gonadogenesis in this species is compared with that in three macropod marsupials, namely the tammar wallaby (Macropus eugenii), the bettong (Bettongia gaimardi) and the potoroo (Potorous tridactylus).

In order to base the comparison on first-hand experience gonadogenesis in the mouse has been re-examined at both the light and electron microscopic levels.

The accounts of ovary development in the bettong and the potoroo are here given for the first time. In the latter species this includes also a histochemical and quantitative study.

Regarding the second objective, the project seeks to throw light on the following problems and events:

i) The origin of the gonadal blastema.

ii) The origin of the follicle cells (granulosa cells).

iii) The origin and possible role of the interstitial gland tissue which occurs in abundance in potoroo ovaries but is not found in all mammals.

iv) The mode of formation, fate and possible significance of the polyovular follicles which likewise do not occur in all mammalian species but are found in large numbers in the immature ovaries of the potoroo.

v) The presence and time of appearance of steroid dehydrogenase

enzyme ($\Delta 5$, 3β HSD) in the marsupial ovaries. This enzyme plays a vital role in the biosynthesis of sex steroid hormones.

vi) The correlation between the histochemical and ultrastructural findings.

vii) Wartenberg (1983) postulates that in the rabbit an interaction between the darkly stained mesonephric cells and the light mesothelial cells initiates gonadal development as well as sex differentiation. Although he did not study the very early stages of gonadal development in mouse embryos, he suggests that a similar situation to that in the rabbit prevails in the mouse. Since such observations have not been reported for the mouse, an investigation of the relevant stages of gonadogenesis to test this hypothesis is justified.

1.2. FORMATION OF THE GONADAL RUDIMENT

The study of gonad development in mammals goes back as far as 1870 when Waldeyer published his monograph on mammalian oogenesis. Most of the studies on gonadal differentiation have been on eutherians (mouse: Simkins, 1923; Brambell, 1927; Upadhyay et al., 1979 & 1981; rat: Simkins, 1923; Torrey, 1945; Mittwoch et al., 1969; Jost, 1973; Merchant, 1975; rabbit: Van Winiwarter, 1900; Gruenwald, 1942; sheep: Zamboni et al., 1981). As far as the marsupials are concerned, the development of the gonad has been reported in five species of marsupials (Tyndale-Biscoe & Renfree, 1987): Didelphis virginiana: Moore, 1939; Morgan, 1943; Dasyurus viverrinus: Fraser, 1919; Ullmann, 1984b; Perameles nasuta & Isoodon macrourus: Ullmann, 1981a; and Macropus eugenii: Alcorn, 1975.

Even though the morphological events of gonadal differentiation

in mammals have been the subject of numerous studies, important aspects of the process remain to be elucidated.

1.2.1. Primordial Germ Cells

The indifferent gonad consists of germ cells and blastema cells invested by mesothelium (coelomic epithelium).

The origin and early development of the primordial germ cells (PGCs) in mammals have been the subject of interest and controversy for a considerable period of time. According to Waldeyer (1870) the mesothelium gives rise to the PGCs, a view which predominated for many years (Kingery, 1917; Simkins, 1923 & 1928; Hargitt, 1926; Brambell, 1927). On the other hand, Weismann (1885) maintained that the PGCs had an extragonadal origin. Weismann's idea subsequently found support in both experimental (Everett, 1943; Mintz, 1957) and histochemical studies (McKay et al., 1953; Chiquoine, 1954).

It is now well established that the PGCs of most mammalian embryos have an extragonadal origin (Zuckerman & Baker, 1977). However, the exact site of their formation is still not known (Wylie et al., 1986). Some investigators report that the PGCs are first seen in the yolk sac endoderm (Everett, 1943; Witschi, 1948; McKay et al., 1953; Chiquoine, 1954; Mintz, 1957; Franchi & Mandl, 1962; Jost, 1973; Guraya, 1977; Byskov, 1986). Recent experiments suggest that the PGCs derive from the ectoderm near the posterior end of the primitive streak (Wylie et al., 1986).

From their site of origin the PGCs migrate along the hindgut, up the dorsal mesentery; from there they pass laterally, in the mesenchyme, to the region of the presumptive gonads. The mechanism involved in their translocation is not yet known (Byskov, 1981; Wylie, 1981). Two ideas have been suggested in the literature - namely active amoeboid movement (Witschi, 1943; Blandau et al., 1963; Zamboni &

Merchant, 1973; Spiegelman & Bennett, 1973; Clark & Eddy, 1975; Fujimotto et al., 1977) and passive movement by tissue in which the PGCs are embedded (Jeon & Kennedy, 1972).

Witschi (1943) believes that the PGCs are attracted to the putative gonads by chemotaxis though he presents little evidence for it. Recently it has been suggested, from experiments on amphibia, that the substrate plays an important role in the migration of PGCs (Wylie et al., 1986). These authors propose that chemotaxis is a mechanism which imposes directionality and polarity on migrating anuran PGCs.

The morphological characteristics of PGCs in mammals have been described in the mouse (Brambell, 1927; Chiquoine, 1954; Odor & Blandau, 1969; Zamboni & Merchant, 1973; Clark & Eddy, 1975), rat (Eddy, 1974; Merchant, 1975), pig (Pelliniemi, 1975), tammar wallaby (Alcorn, 1975), bandicoot (Ullmann, 1981b) and man (Gondos & Hobel, 1971; Kellokumpu-Lehtinen & Soderstrom, 1978).

Several criteria, both morphological and histochemical, have been used by investigators to identify the migrating PGCs. Such PGCs are described as being larger than the surrounding somatic cells, with large vesicular nuclei and several prominent nucleoli. In some mammalian species the nuclear envelope is characterised by the presence of a rim of heterochromatin (mouse: Clark & Eddy, 1975; tammar wallaby: Alcorn, 1975; bandicoot: Ullmann, 1981b) but this arrangement of the heterochromatin disappears once the PGCs become settled in the gonadal ridges. On the other hand, several studies on rodent PGCs (rat: Eddy, 1974; mouse: Merchant & Zamboni, 1973; Spiegelman & Bennett, 1973) did not reveal the presence of this feature.

The presence of alkaline phosphatase has been used as a marker for distinguishing the PGCs from the surrounding tissues in mouse (Chiquoine, 1954) and human embryos (McKay et al., 1953). However, the

PGCs lose such enzyme activity upon arrival in the gonadal ridges.

Alcorn (1975) working on the tammar wallaby, sexed his early embryos and was able to distinguish genetically between the males and females. He observed that the migratory female and male PGCs showed sexual dimorphism in the arrangement of their nucleolus-associated heterochromatin. Alcorn (1975) states that the nucleolus of some of the female PGCs is characterised by the presence of a large, cap-like, darkly staining heterochromatin body concentrated around one pole of the nucleolus, a feature lacking in male PGCs. In the latter, there are a number of small perinucleolar masses of heterochromatin. However, Alcorn (1975) failed to find the nucleolus-associated heterochromatin in some female PGCs (presumably due to the unfavourable plane of sectioning). After the completion of migration, this characteristic arrangement disappears from the female PGCs and a similar arrangement to that of the male heterochromatin becomes established.

In both eutherians (Zamboni & Merchant, 1973) and marsupials (Alcorn, 1975; Ullmann, 1981b) the PGCs have been observed to undergo mitosis during migration.

Several criteria such as the shape of mitochondria, Golgi complex and endoplasmic reticulum (ER) have also been used to distinguish the PGCs from other cell types. The PGCs possess large numbers of ribosomes, a few spherical mitochondria with a few cristae and a well developed Golgi body; the latter consists of one or two stacks of parallel flattened cisternae. Authors give divergent descriptions of the ER in migrating PGCs. According to Jeon & Kennedy (1973) the rough ER is abundant. Zamboni & Merchant (1973) state that it consists of long and branching cisternae, while Spiegelman & Bennett (1973) find only a few profiles of granular ER in the form of short flattened

cisternae.

The structure of PGCs at the settlement stage is much simpler than during the migratory phase (Zamboni & Merchant, 1973; Clark & Eddy, 1975). The organelles, which were present during the migratory period and were presumably involved in metabolic exchange between the germ cells and the surrounding tissues, have decreased in number or disappeared altogether. For instance, the ER decreases in amount (Zamboni & Merchant, 1973).

In bandicoots, the PGCs possess a few rounded mitochondria with transverse cristae, scattered Golgi elements, smooth and coated vesicles; the smooth vesicle may contain an electron opaque substance (Ullmann, 1981b). The PGCs in the bandicoot (Ullmann, 1981b) were found to be less electron dense than in the mouse (Clark & Eddy, 1975).

Several other inclusions are also described in the PGCs. Membrane-bound electron dense bodies or "granules" or "vesicles", either singly or in groups, are observed in the PGCs of different species (mouse: Odor & Blandau, 1969; Zamboni & Merchant, 1973; Clark & Eddy, 1975; rat: Eddy, 1974; pig: Pelliniemi, 1975; bandicoot: Ullmann, 1981b). The nature and significance of these dense bodies is still unknown, though it has been suggested that they may play a role in the meiotic process, as it was found that their number in the PGCs changes before, during and after the beginning of meiosis (Byskov et al., unpublished observations, from Byskov, 1986). Although these bodies were not detected in other cell types in the mouse (Clark & Eddy, 1973; Jeon & Kennedy, 1973), their presence was not restricted to the PGCs in bandicoots, where Ullmann (1981b) observed similar structures also in the somatic cells of the gonadal ridges.

Another cytoplasmic inclusion, the nuage, which is considered to be unique to PGCs is found in the rat (Eddy, 1974) and man

(Kellokumpu-Lehtinen & Soderstrom, 1978; Gondos & Hobel, 1971; Szollosi et al., 1972). The nuage consists of a spherical mass of fibrous or granular material and is closely associated with the mitochondria or the nucleus (Kellokumpu-Lehtinen & Soderstrom, 1978).

In 1892 Weisman put forward a hypothesis now known as the Germ Plasm Theory which states that germ plasm located in the egg determines the germ cell lineage. The germ cell specific polar granules of dipteran insects and the germ plasm of anuran amphibians are known to contain RNA (Eddy, 1975). The nuage in mammals is similar to these inclusions (Wylie et al., 1986), all of which have been considered to be germ cell determinant (Kellokumpu-Lehtinin & Söderstrom, 1978). Wylie et al. (1986), however, throw doubt on this interpretation. They state that recent experiments on the mouse and rat were performed in order to identify germ-line specific antigen markers in or on the surface of PGCs. These experiments were done to determine the time when PGC-specific molecules appear and to trace the origin of the PGCs. It was concluded from these studies that a close relationship exists between some teratocarcinoma cells and migrating PGCs. The latter were found to react with several monoclonal antibodies that were raised against teratocarcinoma and embryonal carcinoma cells. Wylie et al (1986) state that several antibodies react with many cells in the early embryo, disappear during the post-implantation stage and are re-expressed on PGCs during their migration.

A recent surprising observation has been the presence of rudimentary cilia in the PGCs of fetal pig gonads (Byskov et al., unpublished observations - quoted from Byskov, 1986).

Although the PGCs are found in the vicinity of the mesothelial cells, they are not intermingled with them (Everett, 1943; Chiquoine,

1954; Zamboni & Merchant, 1973).

There are contradictory views as to the necessity of the PGCs for the initiation of gonadal ridge development. Some authors claim that the formation of gonadal ridges is independent of PGCs (Merchant, 1975; Ullmann, 1981b). Others hold that the presence of PGCs is necessary for the development of the gonad (Ohno, 1967; Alcorn, 1975).

Merchant (1975), working on rat embryos, eliminated the PGCs before they reached the gonadal ridges using a drug called Busulphan. Although he states that his method does not result in the degeneration of all PGCs before their arrival in the gonadal ridges, he believes that the formation of the gonad can occur in the absence of PGCs or with only a few of them. Moreover, it was found that in bandicoots the gonadal ridges develop before the arrival of the PGCs and it was therefore concluded that their formation was independent of PGCs (Ullmann, 1981b).

On the other hand it is reported that in the tammar wallaby the arrival of PGCs in the vicinity of the mesothelium, between the dorsal mesentery and the mesonephroi, initiates the development of the gonad and the proliferation of the blastema (Alcorn, 1975).

1.2.2. THE BLASTEMA

The exact origin of the blastema is still at present a topic of controversy (Upadhyay et al., 1979; Ullmann, 1981a; Wartenberg, 1981; Byskov, 1986). Some authors (Allen, 1904; Kingery, 1917; Brambell, 1927; Everett, 1943; Bookhout, 1945) derive it from the mesothelium of the gonadal ridges, others from the mesonephroi (Byskov, 1973; Upadhyay et al., 1979; Zamboni et al., 1979 & 1981). The gonadal blastema has also been attributed a dual origin, from the mesothelial and mesonephric cells (Gruenwald, 1942; Torry, 1945; Witschi, 1951; Merchant, 1979; Pelliniemi, 1975, 1979; Byskov, 1982; Wartenberg,

1983). All of the above studies are on eutherian mammals; the only observations on the origin of the blastema in marsupials are those of Alcorn (1975) and Ullmann (1981a, 1984b) for the tammar wallaby, the bandicoots and the native cat respectively.

Some investigators, who hold the first view, describe the formation of the blastema to be a result of a single continuous proliferation of the mesothelium in the form of irregular masses of cells (Kingery, 1917; Brambell, 1927).

The mesonephros is the second consecutive kidney to appear in vertebrate development. It arises posterior to the pronephros and becomes the functional kidney of adult anamniotes. In amniotes it degenerates, except for a part connected to the testes, and is replaced by the metanephros as the functional kidney in the adult. In some species, for instance the mouse, the mesonephroi are rudimentary and lack glomeruli; while in others, as in the rabbit, sheep, pig and human, they are well developed and fully functional during fetal development. It has been suggested that the mesonephroi contribute to the formation of the gonadal blastema, irrespective of their own differentiation and functional state (Wartenberg, 1981). This author believes that the mesonephroi participate in gonadal development by segregating cells either from the intermediate mesoderm, the blastema of the nephrogenic cords or from the developing malpighian corpuscles.

Upadhyay et al. (1979, 1981), working on mouse embryos, believe in the mesonephric origin of the blastema. They state that owing to the rudimentary organization and lack of conventional glomeruli, the mesonephroi appear hardly capable of any excretory activity and that they are involved in gonadal development immediately upon attaining their highest degree of differentiation, at eleven days of fetal life. They found that the blastema cells derive from the ventral terminations of the mesonephric tubules at regions where the basal

lamina becomes discontinous or is completely lacking.

Added support for a mesonephric origin of the blastema cells is given by the presence of mitotic activity within the mesonephric tubules and within the cells derived from them. Moreover, according to Upadhyay et al. (1979), the mesonephric cells which are involved in this migration from the wall of the mesonephric tubules are characterized by the presence of numerous pleomorphic lysosome-like bodies. They suggest that these organelles are probably required for the enzymatic digestion of the basal lamina of the mesonephric tubules. Further support for Upadhyay et al's (1979) belief comes from ultrastructural observations which show a similarity between the somatic cells of the gonads and the mesonephric cells present in the terminal segments of the mesonephric tubules. In both there is a decreased affinity for toluidine blue, the nuclei are large and identical, while the cytoplasm contains large numbers of small, rod shaped, dense mitochondria. This configuration of the mitochondria in the cytoplasm of the mesonephric cells in the terminal segments of the tubules, located just dorsal to the gonadal ridges, differed from those of the more dorsal segments (Upadhyay et al., 1979).

Upadhyay et al. (1979) find no evidence for a mesothelial cell contribution to the gonadal ridges.

In his description of gonadal differentiation in mouse embryos, Wartenberg (1981) states that the S-shaped mesonephric tubules represent the most advanced stages of nephrogenic development. According to him, some of the mesonephric cells which are darkly stained, fibroblast-like in shape and contain lysosomal granules, may also be traced into the gonadal ridge blastema.

Greenwald (1942) believes in the dual origin of the blastema. According to him the mesothelium lacks a basement membrane, with no

line of demarcation between it and the underlying tissue. Witschi (1951) holds the same view and reports that the embryonic gonad consists of an outer layer, the cortex and an inner layer, the medulla. He proposes that the cortex derives from the mesothelium, while the medulla has a mesonephric origin.

The above investigators report on the origin of the blastema cells in species having rudimentary mesonephroi. Other studies are available in the literature describing the formation of the blastema in species having prominent well developed mesonephroi (tammar wallaby: Alcorn, 1975; pig: Pelliniemi, 1976; sheep: Zamboni et al., 1979, 1981).

Pelliniemi (1976), working on pig embryos, distinguished two regions within the gonadal blastema: the outer part, consisting of primitive cords, and a central region designated as the blastema proper. The former is continuous with the mesothelium. On the other hand, the blastema proper consists of irregularly organized cells. The outer region is believed to have a mesothelial origin while the blastema proper has been attributed a dual origin, from mesothelial and mesenchymal cells.

Pelliniemi (1976) presents evidence to support the mesothelial origin of the outer region. He observed that the mesothelial basement membrane is continuous with the cords except in the deepest layer where the cord pattern disappears. Moreover, this author holds that an increase in cell height is the first morphological sign of a starting epithelial differentiation. From the observation of columnar mesothelial cells being very active in mitosis, Pelliniemi (1975) concludes that these cells contribute to the formation of the gonadal blastema. Further support for a mesothelial origin comes from the morphological resemblance between the mesothelial cells and the blastema (according to him the primitive cords and the blastema

proper).

Zamboni et al. (1979, 1981) suggest that the giant nephron, situated in the proximal region of the mesonephros participates in the development of the blastema in the sheep. In this species, the caudal half of each mesonephros consists of nephrons while the cranial half is entirely occupied by a single nephron and numerous tubules. The giant nephron is located dorsal to the gonadal ridge.

Zamboni et al. (1979) distinguish two kinds of cells, the mesanglial and epithelial cells, which leave the giant glomerulus for the gonadal ridges. They postulate active amoeboid movement of the mesonephric cells in reaching the gonadal rudiment. These authors find that these migrating cells show irregular profiles and have numerous pseudopodia. During their migration to the gonadal ridges, these mesonephric cells grouped together to form clusters. Later on, at the settlement stage, they are randomly dispersed and become closely associated with the PGCs.

Zamboni et al. (1981) report that the caudal nephrons undergo regression while the giant glomerulus loses its Bowman's capsule. Subsequently, the glomerular cells of this giant nephron segregate and move ventrally into the gonadal ridges where they multiply, forming the gonadal blastema.

The blastema has been derived from mesonephric mesenchymal cells in several species of marsupials (opossum: Fraser, 1919; brush-tailed possum: Fraser, 1919; bandicoot: Fraser, 1919; Ullmann, 1981a; native cat: Fraser, 1919; Ullmann, 1984b; tammar wallaby: Alcorn, 1975). In the bandicoot, however, Ullmann (1981a) reports that the mesothelium also contributes to blastema cells formation.

In the tammar wallaby, Alcorn (1975) reports that mesenchymal cells migrate from the ventral margin of the mesonephros, close to the

mesonephric glomeruli. He states that the mesenchymal cells migrate across the gonadal ridges below the mesothelium and when they divide they produce condensed cell cords (the central somatic blastema). The blastemal core is first formed anteriorly and its organization proceeds posteriorly. According to him the blastema cells are small, variable in shape from ovoid to elongate with eosinophilic cytoplasm. Their nuclei are small, occasionally dumb-bell shaped, the nucleolar chromatin is granular, while the nuclei are small, prominent and subcentric. Although Alcorn (1975) states that the first sign of gonadal ridge development was an increase of height in the more or less cuboidal cells of the mesothelium, he found no contribution from this source to the central somatic blastema.

Wartenberg (1981, 1983) discusses the problem of somatic cell origins by comparing development in different mammalian species. He distinguishes two cell types on the basis of staining properties in the gonadal blastema of the rabbit (his light and dark cells). He takes this as his basis for a belief in the dual origin of the blastema, from mesothelial and mesonephric cells.

According to Wartenberg (1983), the two cell types which are believed to constitute the gonadal blastema, that is the dark mesonephric cells and the light mesothelial cells, not only have different origins but different functions. He proposes that these two cells have different effects on the PGCs. He states that the mesonephric cells stimulate both the mitotic and the meiotic activities of the PGCs. In contrast, the mesothelial cells are postulated to inhibit the mitotic and the meiotic activities of the PGCs.

1.3. GONADAL SEXUAL DIFFERENTIATION

The indifferent gonads form on each side between the dorsal mesentery and the mesonephroi. Previous authors have recognized two regions in the developing gonads of mammals, an outer zone, the cortex and an inner zone, the medulla.

There is no unanimity in the literature regarding the process of sexual differentiation of the gonads. Several theories have been proposed: the first one suggests that the proliferation of the mesothelium of the gonads, forming the sex cords, results in gonadal sexual differentiation (Brambell, 1927). Furthermore, the differentiation of the testes involves the regression of the cords in the cortex and the persistence of the medullary ones, while the reverse process characterizes ovarian development.

A second theory put forward by Witschi (1931), postulates cortico-medullary antagonism to account for sexual differentiation. He suggests that the cortex derives from the mesothelium and produces a substance called cortexin while the medulla originates from the mesonephros and secretes medullarin. In the presumptive female the cortexin inhibits the development of the medulla and stimulates the development of the cortex. Medullarin, on the other hand, stimulates the development of the medulla and causes regression of the cortex in the genetic male.

A third theory postulates that sexual differentiation might depend on the inductive influence exerted by mesonephric structures (Alcorn, 1975; Merchant-Larios, 1979; Upadhyay *et al.*, 1979; Zamboni *et al.*, 1979; Byskov, 1986).

Yet another explanation for gonadal sexual differentiation has been suggested by Wartenberg (1981, 1983). As has been mentioned before, this author believes in the dual origin of the blastema, from

the mesothelium and the mesenophroi. He proposes that sex differentiation of the gonads is based on the cellular interaction between these different cell types (see Discussion - Chapter 2).

In eutherian and metatherian mammals, the indifferent gonads become sexually recognizable when the testes start to differentiate in genetic males (Jost, 1970; Tyndale-Biscoe & Renfree, 1987).

It was observed that the differentiation of the indifferent gonads into testes appears to occur as a result of autodifferentiation of the blastema cells (Jost et al., 1973; Ullmann, 1981a). In the rat for instance, the differentiation of the indifferent gonads into testes occurs suddenly, with the putative Sertoli cells swelling and their cytoplasm clearing. They then become associated with each other, enclosing the germ cells to form the primary sex cords.

In the bandicoots, the primary sex cords in the testis differentiate randomly and are secondarily displaced to a peripheral position where they form a coherent zone just below the tunica albuginea (Ullmann, 1981a).

The differentiation of the gonad in the genetic female occurs somewhat later.

1.3.1. DIFFERENTIATION OF THE OVARY

Ovary development has been studied in several species of eutherian mammals (mouse: Kingery, 1917; Brambell, 1927; Peters, 1967 & 1969; Odor & Blandau, 1969; Upadhyay et al., 1979; Zamboni et al., 1979; rat: Cowperthwaite, 1925; Torrey, 1945; hamster: Greenwald & Peppler, 1968; Weakly, 1967; rabbit: Allen, 1904; Peters, 1965; Gondos, 1969; Gondos & Zamboni, 1969; Deanesly, 1975; sheep: Zamboni, 1979; pig: Allen, 1904; Bookhout, 1945; Black & Erickson, 1965; Pelliniemi, 1975b; guinea pig: Deanesly, 1975; cattle: Ohno & Smith, 1963 & 1964; Erickson, 1966; human: Ohno & Smith, 1965; Pelliniemi,

1979; Baker & Scrimgeour, 1980; Motta & Makabe, 1982). Only a few studies are available on ovarian development in marsupials (opossum: Nelson & Swain, 1942; Morgan, 1943; tammar wallaby: Alcorn, 1975; bandicoot: Ullmann, 1981b).

In the presumptive ovary, the PGCs enter mitotic division and are then called oogonia. After ceasing division, the oogonia enter meiosis and become oocytes (Byskov, 1981).

The basic event after female sex differentiation is the onset of meiosis (Wartenberg, 1981). The stage of development at which meiosis starts in the putative ovaries is species dependent (Grinsted, 1981; Wartenberg, 1981).

In those species with immediate meiosis, the oogonia enter meiosis simultaneously or shortly after the onset of sexual differentiation, as in the case of the mouse, rat and man (Byskov, 1984; 1986). Before and at the time when meiosis starts, the ovaries of these species are compact organs consisting of uniformly distributed germinal and blastemal cells.

On the other hand, in species with delayed meiosis a long period separates gonadal sex differentiation from the onset of meiosis, as in the case of the pig, cow and sheep (Hoyer et al., 1981). In these species, morphological gonadal sex differentiation precedes the onset of meiosis in the ovary. That is to say, before meiosis starts the female germ cells become enclosed in cell nests resembling testicular cords (Grinsted, 1981). However, the female sex cords differ from the latter by being larger and less organized.

It is believed that the onset of meiosis depends on the relative concentration of two postulated diffusable substances, a meiosis inducing substance (MIS) and a meiosis preventing substance (MPS). It is suggested that the initiation of meiosis is triggered by a MIS which is secreted by the mesonephroi or their derivatives. On the

other hand, it is also held that meiosis does not proceed in the cord enclosed germ cells, due to the action of MPS present within the cords (Byskov, 1981).

The chemical nature of MIS and MPS is not known. However, they are unlikely to be steroids, since application of different steroids to the developing gonads did not induce or prevent the onset of meiosis in the prospective males and females respectively (Byskov, 1981). Baker and Neal (1973) observed that oogonia enter meiotic prophase in the absence of gonadotrophic hormones.

The two kinds of ovarian development (that is, undergoing immediate or delayed meiosis) deviate not only in the time of the onset of meiosis but also in the time at which sex steroid synthesis begins (Grinsted, 1981). While no significant sex steroids are produced by the newly differentiated ovaries which are characterized by immediate meiosis, high amounts of oestrogens are secreted by those with delayed meiosis (Grinsted, 1981) (see Chapter 4).

By the end of meiotic prophase, the oocytes are enclosed by the adjacent somatic cells (the putative granulosa or follicle cells) to form follicles.

1.3.1.1. The rete system

The presence of cellular cords and tubules extending between the mesonephroi and the developing ovaries has been reported as early as 1870 (Waldeyer, quoted by Upadhyay et al., 1979). These cords and tubules constitute the so-called rete system. In eutherian mammals, some investigators derive these cords from the mesothelium (Allen, 1904; Felix, 1912; Morgan, 1943), others from the mesonophroi (Kingery, 1917; Byskov & Lintern-Moore, 1973; Stein & Anderson, 1979).

In marsupials, the rete has been derived from the core of cells situated within the ridge in front of the gonad rudiment (brush-tailed

possum: Fraser, 1919; virginia opossum: Fraser, 1919; bandicoot: Fraser, 1919; native cat: Fraser, 1919; Ullmann, 1984b; tammar wallaby: Alcorn, 1975).

Fraser (1919) considers this ridge a reduced continuation of the gonadal ridge which could not be distinguished from it at the time when it was first formed. Subsequently, the rete ridge becomes recognized from the gonadal ridge and consists of irregular cords and strands of cells. Fraser (1919) states that mesothelial invaginations in the form of solid cords contribute to the formation of the rete system at later stages of development.

The origin of the rete has also been attributed to the mesothelium of the gonadal ridges in the opossum (Morgan, 1943). This author holds that invagination of the mesothelium in the rete region results in the formation of the latter.

Different nomenclatures have been proposed by authors in describing the rete system. According to Byskov and Lintern-Moore (1973), 3 parts can be distinguished in the rete system of the mouse: the intraovarian rete, the connecting rete and the extra-ovarian rete. They consider the intraovarian rete as a system of cords with open connection with the oocytes and follicles while the extra-ovarian rete consists of convoluted tubules in the periovarian tissue; the connecting rete is the region which joins the intra- and extra-ovarian parts of the rete system.

1.3.1.2. Follicle or granulosa cells

The origin of the follicle cells is controversial (Deanesly, 1975; Byskov, 1975; Upadhyay et al., 1979; Linten-Moore et al., 1981; Ullmann, 1981b). Some authors derive them from the mesothelium of the gonadal ridges (Brambell, 1927; Everett, 1943; Franchi & Mandl, 1962; Gondos, 1969; Motta & Makabe, 1982); others give them a mesonephric

origin, deriving them either from the mesonephric tubules (Upadhyay et al., 1979; Zamboni et al., 1979) or from the rete system, which has been considered a derivative of the mesonephros (Byskov & Linten-Moore, 1973; Byskov, 1978; Stein & Anderson, 1979). The granulosa cells have also been derived from the central somatic blastema by Odor and Blandau (1969), Alcorn (1975) and Ullmann (1984a) or from the stroma by Peters and Pedersen (1967), Peters (1969) and Deanesly (1970). The latter investigators appear to use the term stroma to describe completely undifferentiated cells while it is more usual to restrict the term to differentiated connective tissue (Abercrombie et al., 1983). It is presumed that by "stroma" these investigators actually mean the blastema. A dual origin of granulosa cells from the rete ovarii and the ovarian mesothelium has been proposed in the cat, mink and ferret (Byskov, 1975).

Motta and Makabe (1981) studied human fetuses and observed that the mesothelium and the proliferating cords which are related to it, contribute to granulosa cell formation. They state that the cords which are present in the ovarian cortex and the mesothelial invaginations have a common basal lamina. Further support for their contention comes from their observation of steroidogenic features in the cord-mesothelial continuity.

Upadhyay et al. (1979) working on mouse embryos derive the follicle cells from the mesonephric tubules. They believe that septae of connective tissue invade the ovarian cell mass and break it into ovigerous cords of germinal and mesonephric (pre-follicle) cells. They report that these cords appear as extensions or prolongations of mesonephric tubules and that follicle formation initiates in the innermost (dorsal) region of the ovary and within the ovigerous cords.

Stein and Anderson (1979) studied the development of the rete

ovarii in rat embryos using autoradiography. They believe in a rete contribution to the follicle cells.

Byskov (1975) observed that oocytes are in open connection with the mesothelium of the ovary and with the rete system and thus concludes a dual origin for the follicle cells - from the rete and the mesothelium - in the cat, mink and ferret. She found that meiosis and follicle formation occur first in those areas of the cortex where there is contact between the germ cells and the rete cells and therefore suggests that the first follicles occurring within the ovary are connected to the contact area with the intra-ovarian rete system. According to Byskov (1975) the germ cells, except those situated within the periovarian rete body, are influenced by the rete system which is responsible for the initiation of meiosis.

Peters and Pedersen (1967) studied the origin of follicle cells in infant mouse ovaries to ascertain whether they are newly formed or if they derive from cells that are already part of the ovary at birth. They used a radioactively labelled marker, triated thymidine, which they injected into newborn mice; they then prepared autoradiographs at 1 hour, 3 hours and 7 days after the injection. Peters and Pedersen (1967) found that many stromal cells in the centre of the ovary and between germ cell nests incorporated the radioactively labelled marker at birth. At this stage, the ovaries are characterised by the presence of oocyte nests and they are not yet surrounded by granulosa cells. By day 7 after the injection, at a time when the follicles have developed, both the granulosa cells surrounding the oocytes at various stages of development and the stroma cells are labelled. From their observations, Peters and Pedersen (1967) conclude that the follicle cells originate from stroma cells that are present in the ovary at birth. They believe that the central somatic stroma and the stroma surrounding the oocyte nests become attached to the periphery of the

oocytes and begin to divide forming the granulosa cells.

1.3.1.3. Interstitial tissue

The interstitial tissue (IT) was first described by Pflüger (1863) and given the term interstitial gland by Bouin (1902) (quoted by Harrison, 1962).

The IT has been described in the following eutherian mammals: mouse (Quattropani, 1973; Pehleman & Lombard, 1978), rat (Dawson & McCabe, 1951); ferret (Deanesly, 1970); cat (Kingsbury, 1939); rabbit (Allen, 1904; Mori & Matsumoto, 1970); pig (Allen, 1904; Pelliniemi, 1975b); human (Gondos & Hobel, 1971). Among the marsupials, IT has been reported in the brush-tailed possum (O'Donoghue, 1916) and tammar wallaby (Alcorn, 1975) only.

Different classifications have been used by various authors to describe the IT. While Dawson & McCabe (1951), Mori & Matsumoto (1970) and Guraya (1977) classify it into primary and secondary IT depending on source and sequence of appearance, Mossman and Duke (1973) describe seven types of IT based on origin, location, time of appearance, resemblance to endocrine tissue elsewhere in the body and relation to the reproductive cycle. These are the fetal, thecal, stromal, medullary cord, rete, gonadal, adrenal and adneural types of IT (see Mossman & Duke, 1973). However, some authors do not follow either classification and simply use the term interstitial gland tissue or cells (O'Donoghue, 1916; Deanesly, 1970; Moon & Hardy, 1973; Quattropani, 1973; Alcorn, 1975; Pehlemann & Lombard, 1978).

Mossman and Duke (1973) found that the medullary cord IT is best developed in certain groups of carnivora. The IT is derived by hyperplasia and hypertrophy of the medullary cords and often extends into the cortex where it may connect with cortical cords. The thecal type IT occurs in all eutherian mammals and since it develops from the

cells of the theca interna of atretic secondary and vesicular follicles, it is present as soon as follicular atresia begins (Mossman & Duke, 1973). These investigators describe the fully differentiated thecal type IT of most species as containing very large vacuoles of lipid material.

It is believed that the primary and secondary IT differ not only in their sequence of appearance but also in their origin. The primary IT is the first to appear (Guraya, 1977) and has been derived from different sources. Dawson and McCabe (1951) believe that the primary IT arises from cord-like masses which originate from the granulosa cells and from ingrowing cords of cells from the mesothelium. Mori and Matsumoto (1970) agree with Dawson and McCabe (1951) regarding the origin of the primary IT but observe that the medullary cords also participate in its formation.

Several speculations have been expressed as to the origin of the secondary IT. Some investigators derive it from the medullary stromal cells (Allen, 1904; O'Donoghue, 1916; Gruenwald, 1942; Deanesly, 1970; Quattropani, 1973; Pelliniemi, 1975b; Pehlemann & Lombard, 1978; Ullmann, 1984); others from the theca interna of atretic follicles (Kingsbury, 1939; Dawson & McCabe, 1951; Mori & Matsumoto, 1970; Mossman & Duke, 1973), or the rete cords (Alcorn, 1975).

Allen (1904) states that transformation of the stroma gives rise to the theca interna which later on forms the IT in the rabbit and pig. In the possum, O'Donoghue (1916) reports that IT has to be regarded as a "tissue sui generis" and may be a derivative of modified stroma cells at very early stages. He argues against the thecal origin of IT since he observed that IT in the possum developed before the formation of atretic follicles and even before the oocytes became surrounded by a layer of follicle cells. Moreover, he supports his

view by stating that the theca interna is not well developed in marsupials and lacks similarity with the IT. Alcorn (1975) found that the distribution of IT in the tammar wallaby was similar to that in the brush-tailed possum described by O'Donoghue (1916). Alcorn (1975) observed that in both the IT forms small islands of cells scattered in the medullary stroma in its early stages of development; eventually, the IT comes to occupy most of the ovary and the stroma cells pass in interlacing strands through it.

The IT has been examined at the ultrastructural level in some eutherian mammals and characteristic features distinguish it from the surrounding tissues (Mori & Matsumoto, 1970; Quattropani, 1973; Gondos & Hobel, 1973). Such work has not been reported for marsupials.

The IT has been described as being composed of polygonal cells which possess a typical vesicular nucleus; a nucleolus; and more scattered chromatin than the stroma cells (O'Donoghue, 1916). In the tammar wallaby, Alcorn (1975) defines the IT as secreting cells occurring in tightly packed groups or bundles. The individual cells are polyhedral in shape, with fairly distinct cells membranes. Their nuclei are vesicular, occupy a large proportion of the cell volume and have a subcentric nucleolus. The IT is characterized by the presence of features of well established steroid secreting cells such as: abundant diffuse lipid in the cytoplasm, well developed organelles such as mitochondria and smooth endoplasmic reticulum (ER) (Mori & Matsumoto, 1970; Quattropani, 1973; Gondos & Hobel, 1973; Guraya, 1977). Quattropani (1973) describes the IT cells as possessing tubular smooth ER and rounded mitochondria with tubular cristae. Guraya (1977) states that the IT in the sexually mature ovaries of eutherian mammals undergoes changes in quantity and character associated with the ovarian cycle. He adds that membranes of smooth ER play an important role as sites for enzymes which are vital in the

biosynthesis of sex steroid hormones. The precursor material (cholesterol) which is converted into steroid hormones under the stimulation of gonadotrophin is stored in the lipid droplets. Guraya (1977) proposes that steroid hormones form as a result of interaction between the smooth ER, the lipid droplets and the mitochondria. The latter lie adjacent to the lipid droplets and facilitate the release of their storage material which is passed into the smooth ER, where steroid converting enzymes are located.

It has been suggested that the fate of IT differs in different species. While O'Donoghue (1916) finds that the IT in marsupials does not degenerate but undergoes slight hypertrophy during ovulation, Mossmann and Duke (1973) propose that the IT in eutherians may undergo atresia.

1.3.1.4. Polyovular Follicles

Polyovular follicles (POFs) have been described at various developmental stages in some but not all eutherian species (mouse: Engle, 1927a; Fekete, 1950; Kent, 1960; Tagami & Akimoto, 1978; rat: Davis & Hall, 1950; Dawson, 1951; Kent, 1962 & 1964; Bhavsar, 1976; hamster: Kent, 1958 & 1959; Bodmer & Warnick, 1962; Odor, 1965; Weakly, 1966; ferret: Mainland, 1928; cat: Hartman, 1926; Shehata, 1972; squirrel monkey: Hartman, 1926; Graham & Bradley, 1971; rabbit: Szöllösi, 1978; dog: Hartman, 1926; Telfer & Gosden, 1987; human: Gondos & Zamboni, 1969; Papadaki, 1978) and their presence has been regarded as a developmental abnormality (Kent, 1962). In marsupials, POFs have been reported in the native cat (O'Donoghue, 1912); opossum (Hartman, 1926); tammar wallaby (Alcorn, 1975) and potoroo (Ullmann & Brown, 1983).

There is considerable diversity of opinion in the literature as to the origin, fate and possible significance of POFs (Mossman & Duke,

1973). Several speculations have been proposed. Some authors suggest that the POFs result from a failure of oogonia, which are joined by intercellular bridges, to separate before they become surrounded by follicle cells (Gondos & Zamboni, 1969; Alcorn, 1975). Others believe that isolated but closely apposed germ cells may become surrounded by a common envelope of follicle cells (O'Donoghue, 1912; Hartman, 1926; Mainland, 1928; Mossman & Duke, 1973; Papadaki, 1978; Tagami & Akimoto, 1978).

Gonadotrophin and steroid stimulation have also been related to the incidence of POFs by some authors (Fekete, 1950; Kent, 1958, 1959, 1960, 1962; Bodmer & Warnick, 1962; Graham & Bradley, 1971; Shehata, 1974; Bhavsar, 1976). According to Fekete (1950) the proliferation of the mesothelium (from which she derives the germ cells) is correlated with the oestrous cycle. She suggests that POFs form as a result of abnormal development of the mesothelium which is under hormonal control.

Mainland (1928) reports that POFs in the ferret result from the lack of balance in development between the follicle cells and the connective tissue during follicle formation. That is to say, there are not enough follicle cells for the large number of oocytes.

A very similar interpretation to that of Mainland (1928) is put forward by O'Donoghue (1912) and Hartman (1926) for the native cat and opossum respectively. O'Donoghue (1912) states that it is not possible to believe that POFs with five ova can arise from the division of a single ovum. Moreoever, he suggests that his observation of biovular follicles with 2 equal sized ova argues for their formation from the division of one cell. According to Hartman (1926) the proliferation of the mesothelium results in the formation of ova which he believes migrate into the blastema in the form of strands (Pflüger tubes). He

states that if the number of follicle cells is not enough then groups of oocytes become surrounded by common follicle layer.

In the juvenile rhesus monkey, Mossman and Duke (1973) derive the POFs from the medullary cords (or sex cords). They state that the latter are present in many mammals and they often contain oocytes. Mossman and Duke (1973) suggest that the number of oocytes in the POFs depends on the size of the medullary cords as well as on the number of oocytes within the cords. These authors state that small medullary cords with few ova break up to form uniovular follicles while thick cords made up of several cell layers and containing several oocytes dissociate to give rise to polyovular follicles.

It has been suggested that oestrogen levels affect the incidence of polyovuly either directly, by influencing the ovarian tissues, or indirectly through the pituitary gland (Kent, 1958, 1959, 1962). Kent (1959) found that in immature hamsters the POFs form as a result of a deficiency in the production of oestrogen.

POFs may be found at different stages of development. However, they occur most commonly in primordial follicles (Hartman, 1926; Engle, 1927a; Fekete, 1950; Alcorn, 1975; Bodmer & Warnick, 1962; Tagami & Akimoto, 1978) while few have been reported to have reached the antral stage (O'Donoghue, 1912; Hartman, 1926; Engle, 1927a; Fekete, 1950; Bodmer & Warnick, 1962; Ullmann, personal communication).

The number of oocytes in the POFs of eutherians varies from two to five, biovular and triovular follicles being the most common (Engle, 1927a; Fekete, 1950; Dawson, 1951; Alcorn, 1975; Tagami & Akimoto, 1978). O'Donoghue (1912) found POFs with 5 ova in the ovaries of the native cat while Ullmann and Brown (1983) report the presence of follicles containing up to 10 oocytes in the ovaries of the potoroo. The most numerous oocytes per POF recorded for any mammal is

that of the opossum in which Hartman (1926) observed up to 120 ova in a single follicle.

The size of the ova within the POFs is also variable. They may be of dissimilar or equal size (O'Donoghue, 1912; Hartman, 1926; Engle, 1927a; Fekete, 1950; Dawson, 1951). Engle (1927a) reported that most of the ova in the POFs are of equal size; she found only a single POF with oocytes of dissmilar size in the mouse ovary. The presence of oocytes in the biovular follicles having equal sizes is attributed to division of one cell while the dissimilarity in oocyte size in the POFs is related to either the arrest of growth in one of the oocytes or to their unequal rates of growth (Dawson, 1951).

Oocytes in POFs may undergo meiosis in vitro (Jagiello, 1973) as well as in vivo (Graham & Bradley, 1971). In the opossum, however, Hartman (1926) found that the POFs exhibit neither mitotic nor meiotic divisions.

Some investigators consider the POFs as abnormal and destined to atresia (Hartman, 1926), while others (Davis & Hall, 1950; Bodmer & Warnick, 1962; Bhavsar, 1976) believe that they may be capable of ovulation.

Davis and Hall (1950) studied the relationship between polyovuly and fecundity by comparing the number of new corpora lutea in rat ovaries with the number of embryos found in the uterine horns of the same animal. They observed that the number of embryos was more than the corpora lutea. Accordingly, Davis and Hall (1950) concluded that the follicles release more than one viable ovum.

On the other hand Bhavsar (1976), also working on the rat, found more corpora lutea than embryos in the uterine horns and postulates that these extra corpora lutea may have been formed either from ovulated or unovulated POFs. Bhavsar (1976) suggests that the POFs may

be capable of full development and ovulation but that the ova are not viable for fertilization.

CHAPTER TWO

GONAD DEVELOPMENT IN MOUSE PUPOID FOETUS EMBRYOS

2.1. INTRODUCTION

This chapter describes gonad development in mouse embryos at the light and electron microscopical levels. Since there have been several previous publications in this area, some justification of this study may be appropriate.

1. Although there are numerous studies of gonad development in the mouse, these are largely concerned with the structure and migration of the PGCs (Kirkham, 1916; Kingery, 1917; Everett, 1943; Chiquoine, 1954; Mintz, 1957; Mintz & Russell, 1957; Blandau et al., 1963; Spiegelman & Bennett, 1973; Zamboni & Merchant, 1973; Clark & Eddy, 1975; Zamboni & Upadhyay, 1983); those which concern themselves with the somatic elements of the gonad concentrate on the later stages, that is from day 11 post coitum onwards (Odor & Blandau, 1969; Upadhayay et al., 1979, 1981). Thus although the latter describe gonadal differentiation in mouse embryos, they do not consider gonadal ridge formation but concentrate exclusively on the region of the mesonephroi and their contribution to gonad formation.
2. Secondly, while ultrastructural studies of the earliest stages of gonadal ridge formation in embryos of known chromosomal sex have been carried out on the pig (Pelliniemi, 1976) and human (Pelliniemi et al., 1979) no such studies have been performed on mouse embryos.
3. Thirdly, as already mentioned in section 1.1, there are ambiguities in the literature regarding the origin of the blastema cells.
4. As mentioned in section 1.3, Wartenberg (1983) distinguishes two cell types in the gonadal blastema of the rabbit. On the basis of his observations he believes in a dual origin of the gonadal

blastema. Though he did not study the earliest stages of mouse gonad development, he suggests that on days 12 and 13 of fetal life a similar situation to that in the rabbit occurs and postulates that an interaction between the mesonephric and the mesothelial cells initiates gonad formation as well as sex differentiation in the mouse and rabbit (section 1.3). Since such observations have not actually been reported for the mouse a reinvestigation of gonadogenesis to test this hypothesis is justified.

5. Lastly, as already indicated in section 1.1, this study forms a basis for comparison with the marsupials investigated.

In this study the Pupoid foetus, which is a mutant strain of mouse, first described by Meredith (1965), has been used. The choice of species was largely dictated by availability. Since the known effects of this abnormality are ectodermal and the mutation does not manifest itself till later on in development, that is after the formation of gonads (at 11 days post coitum), the mutation is not expected to interfere with the normal course of gonad development. As controls, the Glasgow hybrid stock of mice were used (Ullmann, 1976).

Gonad development in the mouse has previously been described by Kirkham (1917), Kingery (1917), Brambell (1927), Borum (1961, 1966), Upadhyaya et al (1979, 1981) and Wartenberg (1981, 1983).

All the earlier investigators (Kirkham, 1916; Kingery, 1917; Brambell, 1927) believe in the mesothelial origin of the primordial germ cells. In mouse embryos, the blastema has been derived from the mesothelium of the gonadal ridges (Kingery, 1917; Brambell, 1927), from the mesonephroi (Upadhyay et al., 1979) or from both the mesothelium and the mesonephroi (Wartenberg, 1981, 1983).

As is described in Section 1.3, p. 15, different views appear in

the literature regarding the process of gonadal sexual differentiation. As far as the mouse is concerned, while Brambell (1927) and Upadhyay (1981) believe a mesonephric influence causes gonadal sexual differentiation, Wartenberg (1983) suggests that an interaction between 2 different cell types (mesonephric and mesothelial) initiates this process.

2.2. MATERIALS AND METHODS

2.2.1. Maintenance of the pupoid foetus stock

Mouse embryos carrying the pupoid foetus gene (EPF) were obtained from stock maintained in the Developmental Biology Laboratory, University of Glasgow. The mice were kept at a temperature of 20-22^{o}C, with a regular light:dark cycle of 10:14 hours, a constant supply of water and compound diet pellets (Diet 41: Dixon and Sons, Ware, Herts; or Breeding Diet: Oxoid Ltd., London).

The oestrous cycles of the females were brought into synchrony by prior exposure to bedding material from a male's cage for 2 days, a phenomenon called the Whitten effect (Whitten 1966). A male was then put in with 2-3 females in the afternoon and the latter examined for the presence of vaginal plugs on the following three mornings and evenings. Individual females were identified by characteristic ear clips. Embryos were aged from the day when the copulation plug was detected, which was taken to be day "0" of gestation.

2.2.2. Gonadal preparation

When the embryos were of the required age (9-14 days), the mothers were killed by cervical dislocation and a horizontal abdominal incision made, using scissors and forceps. With the aid of a Wild M5 binocular microscope, the uteri were dissected out and placed in a petri dish containing Tyrode balanced salt solution. The embryos were then freed from the uteri with the use of fine surgical forceps, the

trunks sectioned just behind the forelimbs and the anterior body regions discarded. Using overhead illumination and an objective magnification of x6 or x12 plus fibre optics, the gonads were carefully removed from the embryos, either alone or with the mesonephroi attached; or with the mesonephroi and the body wall, as in the case of 10 day old embryos. Dissection of the relevant parts was too difficult in the 9 day old embryos, so the whole of the caudal body region, posterior to the forelimbs, was processed.

2.2.3. Sexing of embryos

Cytological identification of sex prior to gonadal sex differentiation was performed by the chromosome analysis method (Pelliniemi & Salonius, 1976) or the Barr body technique (Harvey, 1971).

2.2.3.a. Chromosome analysis

Tissue from liver or head was used and treated according to the following procedure:

1) Place the tissue in 10ml of Parker's medium TC 199 (Difco) containing 20% calf serum.
2) Mince and squeeze the cells using surgical forceps and scissors.
3) Add a few drops of 50ug/liter colchicine to the suspension of cells, to stabilize the chromosomes in mitotic metaphase.
4) Incubate at 37^{o}C for 2 hours.
5) Centrifuge at 700 rpm and then discard the supernatant.
6) Add 2.5ml of distilled water.
7) Aspirate and leave in an incubator at 37^{o}C for 20 minutes.
8) Centrifuge at 700 rpm and discard the supernatant.
9) Fix in freshly prepared 1:3 acetic acid:methanol for 15 minutes, aspire and centrifuge at 800 rpm.

10) Discard the supernatant and add fresh fixative. Repeat this procedure three times.

11) Place a few drops of the suspension on a microscope slide at 4°C for a few seconds.

12) Dry and fix on a hot plate at 60°C.

13) Cool and stain the slide with 5% Giemsa or lacto-propionic orcein, for 5 minutes.

The metaphase chromosomes were then examined under the oil immersion objective, photographed and the karyotypes prepared.

2.2.3.b. Barr body or sex chromatin visualization

2.2.3.b1. Rationale for demonstration of the Barr body

Female mammalian cells characteristically have two X chromsomes, while male cells contain one X and one Y sex chromosome. In most mammals one of the X chromosomes in the female becomes inactivated during embryonic development and the condensed mass comes to be located near the nuclear membrane. Since the chromatin material is composed largely of DNA and nucleoprotein the inactivated chromsomes can be visualised by any stain specific for DNA or for chromatin (Humason, 1979).

2.2.3.b2. Technique for demonstration of the Barr body

The amnion was dissected out from the embryos into Tyrode saline and processed according to the following schedule:

1) Fix in several changes of freshly prepared 3:1 methanol:acetic acid for 15 minutes.

2) Remove the fixative and add 60% acetic acid for 30 seconds.

3) Aspirate and place a few drops of the suspension on a clean glass slide.

4) Dry on a hot plate at 60°C.

5) Stain with 5% Giemsa stain for 5 minutes.

6) Rinse in several changes of distilled water.

Slides were then examined under the oil immersion objective for the presence of sex chromatin.

2.2.4. Histological procedures

2.2.4.1. Preparation of sections for light microscopy

2.2.4.1a. Paraffin wax sections

Gonadal primordia or the posterior part of 9-10 day old embryos were fixed in aqueous Bouin's solution for 24 hours at room temperature and processed according to the following scheme:

1) Wash with several changes of 70% ethyl alcohol.

2) Dehydrate in 90% and 95% alcohol for 30 minutes in each.

3) Clear in xylene or toluene for 10-15 minutes.

4) Infiltrate with 50:50 xylene:paraffin wax for 30 minutes.

5) Embed in pure paraffin wax.

Blocks were then sectioned transversally or longitudinally at 4-6µm using a Beck microtome and stained with Mayer's haemalum and eosin using a standard histochemical technique (Humason, 1979) (see Appendix).

Sections were then dehydrated in a series of alcohols (30%, 50%, 70%, 90%, 95%), cleared in histosol and mounted in DPX.

The sections were then examined with a Wild M20 microscope and photographed using Kodak Panatomic x film.

2.2.4.1b. 1µm plastic sections:

Half strength Karnovsky fixative (Karnovsky, 1965)

For 1µm thick sections, the gonads and the posterior part of the embryos were fixed in cold half strength Karnovsky solution (see Appendix) for 2 hours at 4°C and processed as follows:

1) Wash in several changes of sodium cacodylate buffer (see

Appendix).

2) Post fix in 1% osmium tetroxide solution in veronal acetate buffer (see Appendix) for 2 hours at 4°C.
3) Wash in several changes of cacodylate buffer.
4) Dehydrate in 70%, 90%, 95% ethyl alcohols for 30 minutes in each.
5) Dehydrate in absolute alcohol, 3 changes, 30 minutes in each.
6) Dehydrate in absolute alcohol for 15 minutes.
7) Clear in a series of 3 changes of propylene oxide: absolute alcohol (25:75, 50:50, 72:25) for 30 minutes in each.
8) Infitrate with 50:50 propylene oxide:araldite resin for 2 hours.
9) To evaporate the propylene oxide, remove the lids from the glass tubes and leave on stirrer for 24 hours.
10) Embed in araldite resin at 45°C for 2 hours, then at 60°C for 24 hours.

Semithin sections were then cut with glass knives on a Reichert OMU3 ultramicrotome, stained with 1% toluidine blue, mounted in DPX, and examined under the light microscope.

2.2.4.2. Preparation of sections for transmission electron microscopy:

The gonads were prepared and fixed according to the method described in section 2.2.4.1b.

This method of fixation resulted in mitochondria being vacuolated and therefore the following alternative method which was found to give more satisfactory results was used.

Glutaraldehyde phosphate fixative

The specimens were fixed in glutaraldehyde phosphate solution (see Appendix) for 2 hours and treated according to the following schedule:

1) Rinse in 3 changes of rinsing solution (see Appendix) for 10 minutes in each.
2) Post-fix in 40% osmium oxide solution in distilled water and rinsing solution for 2 hours.
3) Add distilled water and leave for 5 minutes.
4) Wash in several changes of distilled water, 10 minutes in each.
5) Dehydrate at room temperature in 30%, 50%, 70%, 90%, 95% ethyl alcohols for 10 minutes in each.
6) Dehydrate in 2 changes of absolute alcohol, 10 minutes in each.
7) Dehydrate in dried absolute alcohol for 10 minutes.
8) Clear in 2 changes of propylene oxide for 10 minutes in each.
9) Infiltrate with 50:50 propylene oxide:resin for 24 hours.
10) Embed in pure resin at 45°C for 1-3 hours then at 60°C for 24 hours.

The blocks obtained by the above two methods were trimmed into four-sided pyramids with a double-edged stainless steel razor blade. Sections were cut, using freshly prepared glass knives, on a Reichert OMU3 ultramicrotome mounted on glass slides, dried on hot plate and stained with 1% aqueous toluidine blue in 1% borax.

Ultrathin sections were then cut on a Reichert OMU3 ultramicrotome, stretched with chloroform, mounted on either coated or uncoated 100-300-G copper grids, stained with 4.4% uranyl acetate and 4.4% lead citrate for 5 minutes in each and examined under the transmission electron microscope (A.E.I. 801).

2.3. RESULTS

2.3.1. 9 day old embryos

The mesonephric (primitive kidney) rudiment consists of undifferentiated masses of nephrogenic cells (Fig. 2.1).

At this early stage, the gonadal primordia cannot be

distinguished as such, but their future location is already indicated by the differentiation of the mesothelium in the region of the presumptive gonads: whereas the mesothelium elsewhere is flattened, in this region it consists of a layer of columnar cells lacking a basal lamina (Fig. 2.3).

The mesothelial cells are connected to each other by junctional complexes such as tight junctions and desmosomes. A few short microvilli project from their free surfaces. The nuclei are elongated with one or more prominent nucleoli. The mitochondria are elongated or spherical with irregularly arranged cristae and may be associated with the rough endoplasmic reticulum (ER). Occasional lipid droplets and Golgi complexes are also found.

A few scattered mesenchymal cells are present internal to the mesothelium.

A few isolated cells, distinguished by having spherical nuclei, more than one nucleoli and pale staining cytoplasm, are found in the mesothelium (Fig. 2.1). Cells having similar characteristics except for being denser are found scattered among the epithelial cells of the hindgut, including its luminal surface, as well as in the mesenchyme (Fig. 2.2a & b). It is presumed, judging by their distribution, that these larger cells are the primordial germ cells (PGCs). Mitotic figures are encountered among the PGCs which are embedded in the mesothelium as well as in those located in the hindgut epithelium. The PGCs which are found in the mesothelium and those leaving the hindgut show pseudopodial processes, suggesting that they migrate by amoeboid movements (Fig. 2.2b).

No differences were noticed between the genetically male and female gonads at this early stage of development.

2.3.2. 10 day old embryos

The elongated mesonephroi, the primitive kidneys, are located

about the mid body level and extend posteriorly through the whole abdominal region along both sides of the dorsal mesentery.

At this stage the gonadal ridges have formed and project into the coelomic cavity medial to the mesonephroi (Fig. 2.5).

The mesothelium, which is irregular in thickness (Fig. 2.5, 2.6, 2.9 & 2.10), exhibits many mitotic figures the spindles of which are at right angles to the gonadal surface.

By this time, the undifferentiated masses of nephrogenic cells have transformed into mesonephric cords which are regular in shape except for those located close to the gonadal ridges. In this region the mesonephric cords have irregular profiles, lack a basal lamina and contain some pyknotic cells (Fig. 2.8).

The PGCs, now clearly recognisable as such, have increased in number within the gonadal ridges, in the mesenchyme as well as in the dorsal mesentery. In the former the PGCs have spherical nuclei and are closely associated with the mesothelial and mesenchymal cells (Fig. 2.7). The PGCs of the gonadal ridges are larger in size than the surrounding few somatic cells and have denser, more vesicular nuclei.

By the end of this stage, that is at day 10.5 of gestation, the mesonephric cords have transformed into mesonephric tubules. The mesothelium has increased in thickness as a result of mitotic divisions and its cells have elongated and vary in their affinity for the toluidine blue stain used. The blastema consists of irregular cells with several prominent nucleoli (Fig. 2.9).

2.3.3. 11 day old embryos

The indifferent gonads appear elongated, transparent and completely attached to the mesonephroi as seen in the dissected specimens.

Although there is no line of demarcation between the mesonephroi

and the gonadal promordia, the latter can be recognised in sections by the density differences in the cells comprising the two regions: the gonads appear denser (Fig. 2.11 & 2.12).

The PGCs can be identified by the same morphological characteristic as at the previously described stage (that is, at day 10 post coitum). The nuclei are spherical and some may be irregular. During this period, the gonad thickens and elongates as a result of the increased number of blastema and germ cells. A few blood capillaries with nucleated erythrocytes invade the centre of the indifferent gonads from the adjacent mesonephroi.

The mesonephric tubules are well developed, some being s-shaped (Fig. 2.13 & 2.14) while others form cords with irregular profiles (Fig. 2.11). This configuration of the tubules and the absence of the basal lamina (Fig. 2.14a & b) suggest that the former contribute to the formation of the gonadal blastema.

At the end of this stage the sex still cannot be distinguished microscopically. However, the putative testes (Fig. 2.16), as judged by chromosomal analysis, appear larger than the presumptive ovaries which retain most of the characteristic features of the indifferent stages (Fig. 2.15). The future testes, on the other hand, are invested with mesothelium made up of columnar and cuboidal cells and a few blood capillaries with nucleated blood cells penetrate the periphery of the rudiment (Fig. 2.16).

In the male, the blastema differentiates into 2 cell types: fibroblast-like cells and larger irregular cells with several nucleoli.

2.3.4. 12 day old embryos

By the beginning of day twelve of gestation, the gonadal sex can be distinguished microscopically.

2.3.4.a. The testicular rudiments

The male gonad rudiments are invested by a regular mesothelium, separated from the underlying tissues by a continuous basement membrane. The mesothelium is composed of a cuboidal layer of cells which in regions is multilayered and very few PGCs are trapped in it. At this stage, a few fibroblast-like cells (the tunica albuginea) are found internal to the mesothelium at the dorso-lateral side of the gonads (Fig. 2.17).

At the end of this stage, that is at 12.5 days of gestation, the male gonad rudiment can be distinguished by the presence of the spermatic vein in the periphery, just internal to the mesothelium.

The larger blastema cells swell, lose their affinity for the stains used and clump together enclosing the germ cells (prespermatogonia) to form seminiferous cords which eventually become surrounded by a basement membrane.

2.3.4.b. The ovarian rudiments

The female gonad rudiments are thinner and more elongated than the testicular rudiments and retain most of the characteristic features of the indifferent stages (Fig. 2.18). The previously irregular cells comprising the mesothelium are now mostly columnar in shape. A few scattered capillaries with nucleated blood cells are found in the rudiments. The PGCs divide to become oogonia.

2.3.5. 13 day old embryos

2.3.5.a. The testicular rudiments

At day thirteen post coitum, the tunica albuginea becomes organised into several layers.

The seminiferous cords are well developed and surrounded by a continuous basement membrane (Fig. 2.19). The male germ cells are

found at various stages of mitotic division and some of them are pyknotic. Synchronous division of germ cells does not characterise the seminiferous cords at this stage of development.

2.3.5.b. The ovarian rudiments

On day thirteen of gestation, the ovarian rudiments are oval with no trace of a tunica albuginea (Fig. 2.20). They are completely attached to the mesonephroi and are made up of a compact mass of germinal and blastema cells which become more vascularized than in the previously described stages. The mesothelial cells are now cuboidal in shape and many oogonia are found among them. Moreover, at this stage, only a few dividing oogonia were encountered and some of them were pyknotic.

At the end of this stage, that is at 13.5 days post coitum, some of the oogonia enter the prophase stage of the meiotic division.

2.3.6. 14 day old embryos

2.3.6.a. The testicular rudiments

During day 14 of gestation the testicular rudiments undergo compaction and are separated from the mesonephroi, except at the hilar region.

The mesonephroi are narrow structures consisting of degenerating mesonephric tubules (Fig. 2.21b). The investing mesothelium is made up of flattened cells (Fig. 2.21a). The majority of the PGCs, which may be binucleate, are now found in the centre of the seminiferous cords.

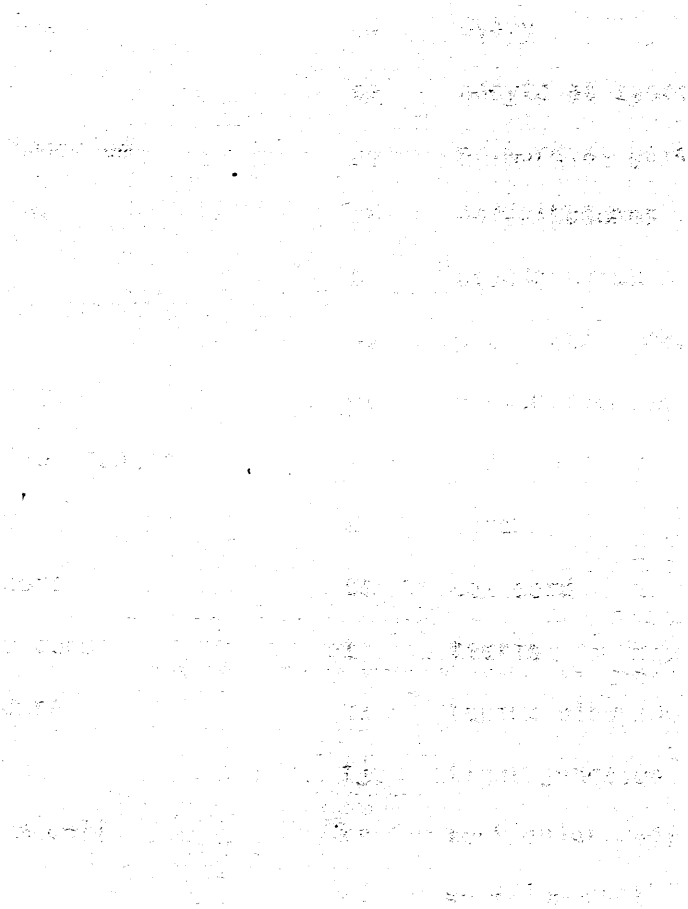
2.3.6.b. The ovarian rudiments

During this stage, the ovarian rudiments increase in size and separate from the mesonephroi, except at the hilar region.

Unlike the previously described stages, at day 14.5 post coitum the rudiments are invested with a somewhat regular mesothelium

separated from the underlying tissue by a discontinuous basement memrbane. The mesothelium is made up of both cuboidal and flattened cells.

Most of the oogonia, except those embedded in the mesothelium, have entered the prophase stages of the meiotic division and are found in groups of morphologically similar cells, suggesting synchronous divisions (Fig. 2.22a & b).



Abbreviations

a	aorta	mst	mesonephric tubule
b	blastema	mt	mesonephric tubule cell
bc	blood capillary	n	nucleus
bL	basal lamina	nc	notochord
c	coelom	nm	nephrogenic mass
d	desmosome	nu	nucleolus
dm	dorsal mesentery	o	oogonium
e	erythrocyte	ol	oocyte at leptotene
er	endoplasmic reticulum	or	ovarian rudiment
G	Golgi complex	ov	ovary
gc	germ cell	oz	oocyte at zygotene
gp	gonadal primodrium	pgc	primordial germ cell
gr	gonadal ridge	pr	polyribosomes
h	hilum	p	pseudopodium
hg	hindgut	pS	pre-Sertoli cell
L	lipid droplet	ps	prespermatogonium
llg	lysosome like granule	r	ribosomes
m	mesothelium	s	stroma
mc	medullary cord	sc	sex cord
msc	mesonephric cord	t	testis
mf	mitotic figure	ta	tunica albuginea
mi	mitochondria	tj	tight junction
mgc	mitotic germ cell	tr	testicular rudiment
ms	mesonephros	Wd	Wolffian duct

For all abbreviations see page 45

Fig. 2.1a. Transverse section through the abdominal region of a 9 day old mouse embryo, to show general structure. (Bar = 100 μm).

Fig. 2.1b. Detail of marked region in Fig. 2.1a to show the gonadal ridge with germ cells (pgc) and the nephrogenic mass (nm). (Bar = 50 μm).

Fig. 2.2a. Transverse section through the hindgut of a 9 day old mouse embryo. Note a primordial germ cell (pgc) adjacent to the lumen of the hindgut (hg). (Bar = 25 μm).

Fig. 2.2b. Note primordial germ cells (pgc) leaving the hindgut (hg) and a mitotic germ cell in the gonadal ridge (gr). (Bar = 25 μm).

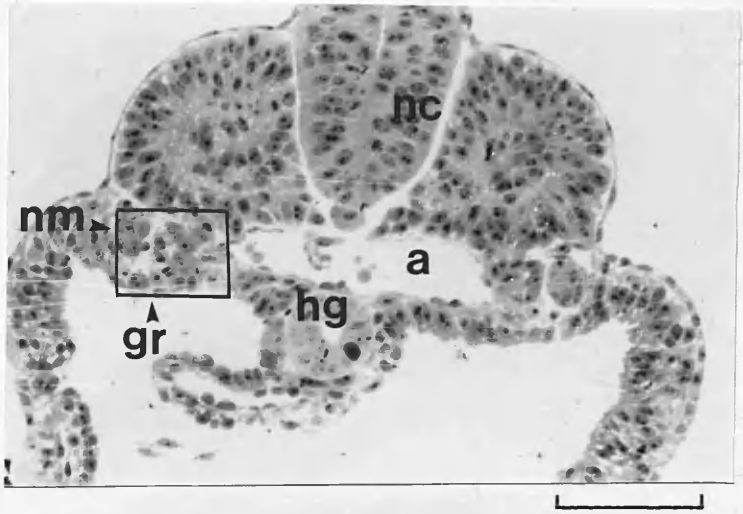


Fig. 2.1a

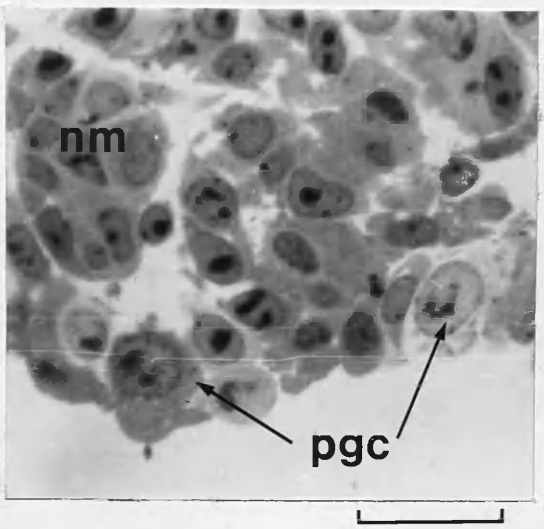


Fig. 2.1b

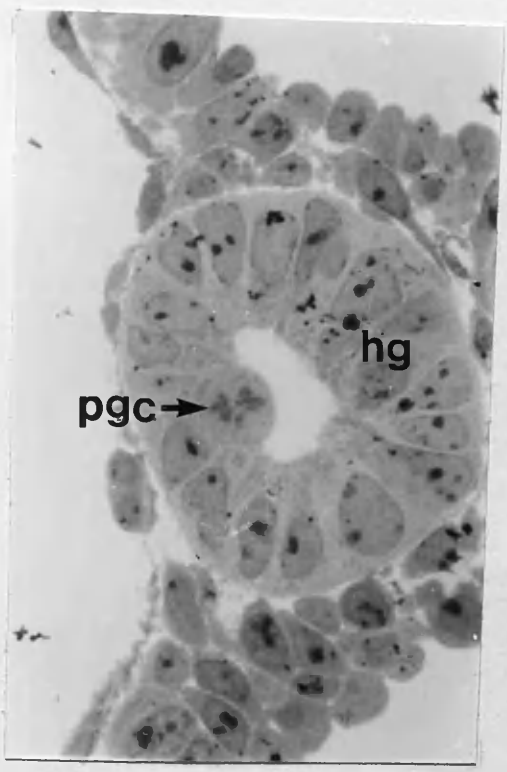


Fig. 2.2a

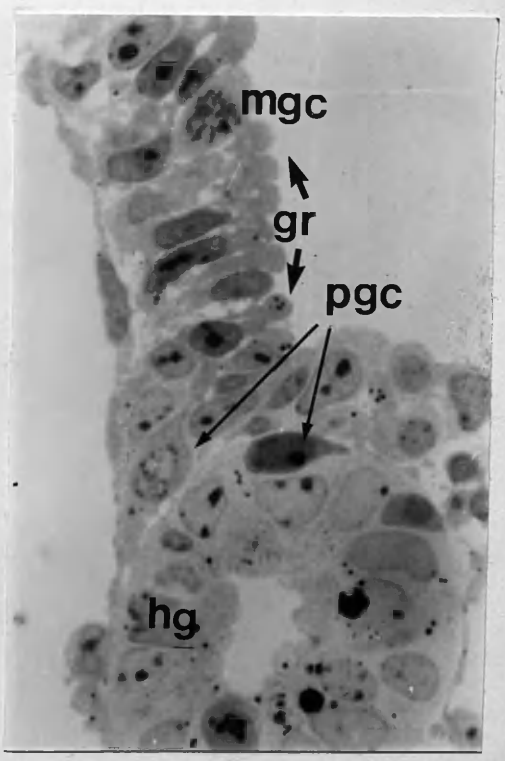


Fig. 2.2b

Fig. 2.3. Electron micrograph through the gonadal ridge of a 9 day old mouse embryo. Note the columnar mesothelial cells (mc) and the discontinuous basal lamina (bl). x 7.9 K.

Fig. 2.4. Electron micrograph through the nephrogenic mass of a 9 day old embryo. x 2.4 K.

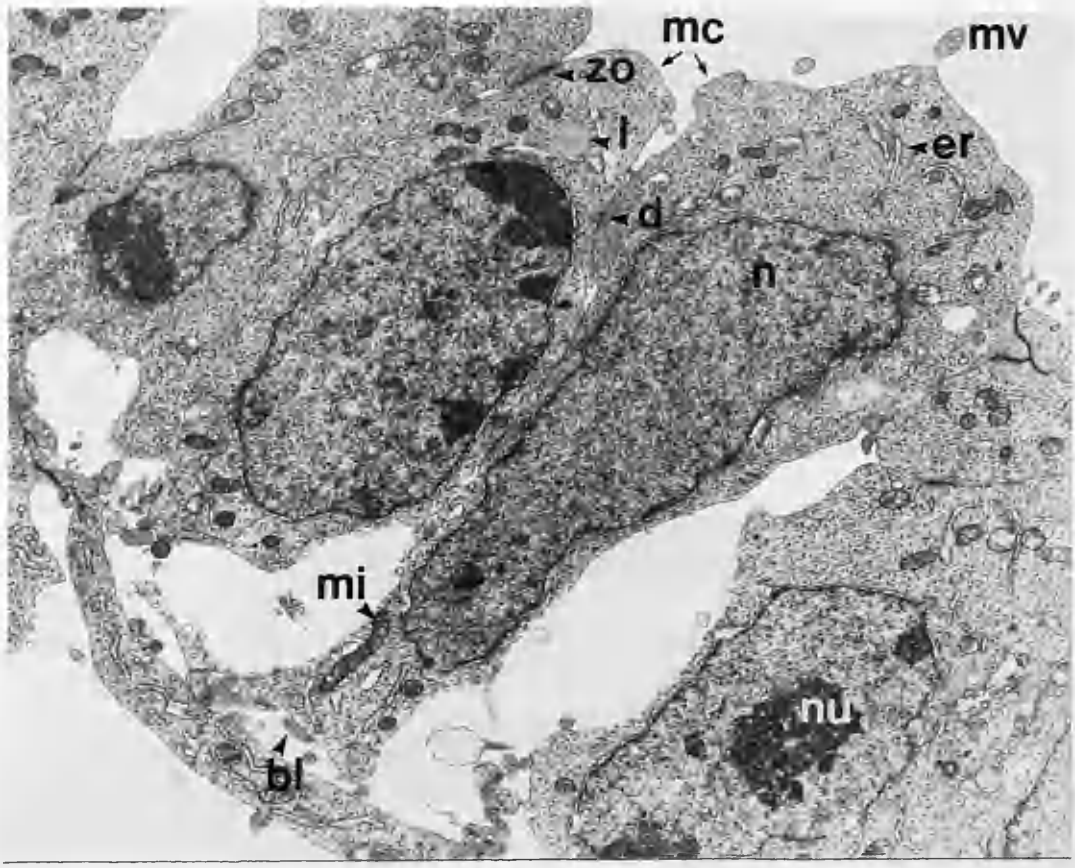


Fig. 2.3

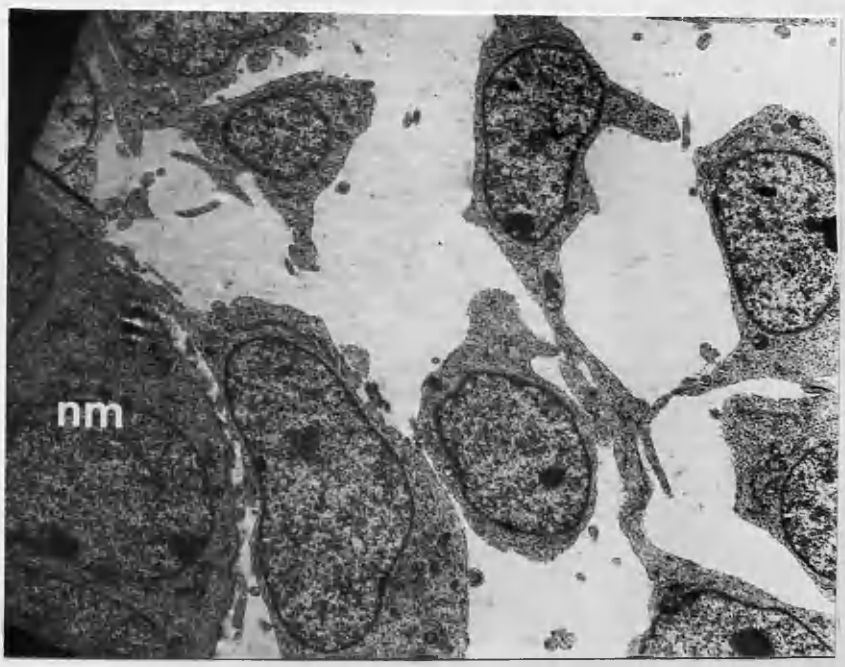


Fig. 2.4

Fig. 2.5. Transverse section through the gonadal ridge of a 10 day old mouse embryo, to show gonadal ridges (gr). (Bar = 100 μm).

Fig. 2.6. Electron micrograph through the gonadal ridge of a 10 day old mouse embryo. Note the lack of a basement membrane between the superficial and deeper lying cells. x 3.75 K.

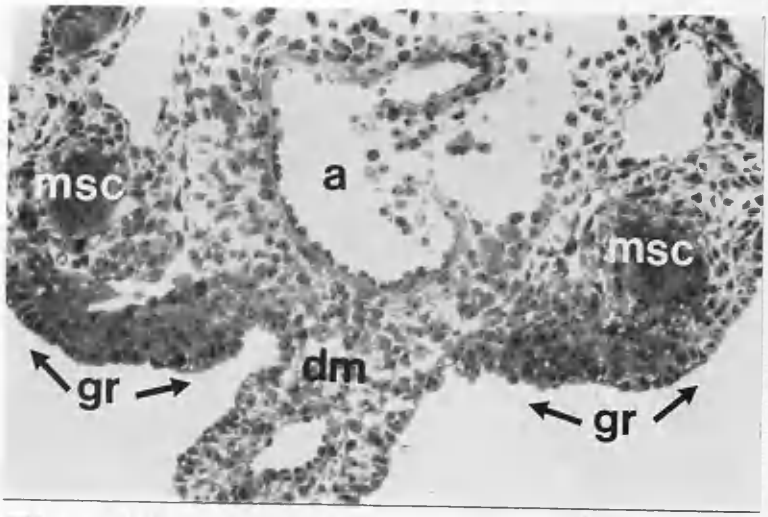


Fig. 2.5

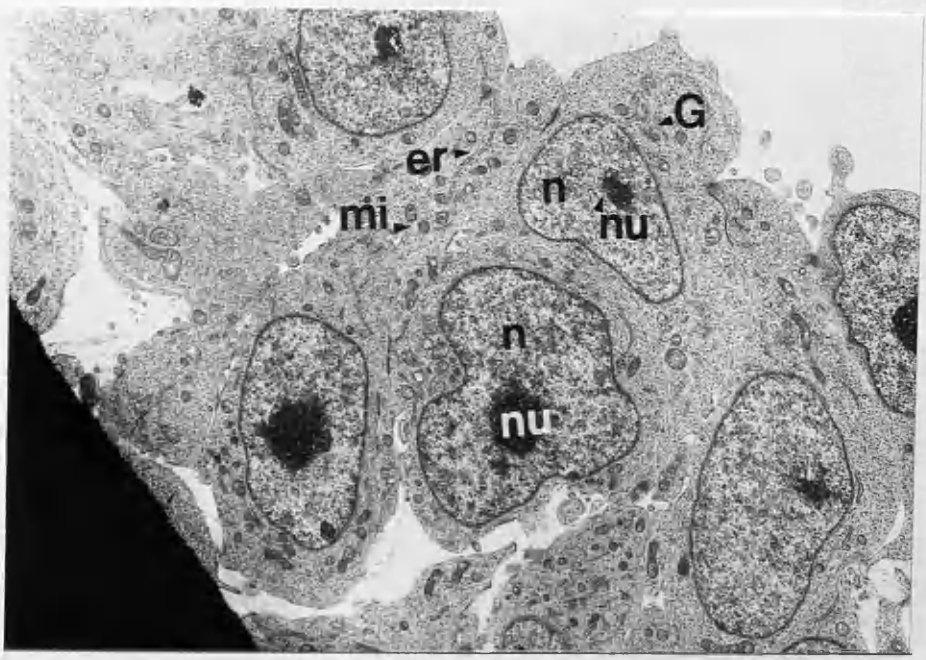


Fig. 2.6

Fig. 2.7a. Transverse section through the gonadal ridge (gr) and the dorsal mesentery (dm) of a 10 day old mouse embryo, showing the distribution of the primordial germ cells (pgc). (Bar = 50 μm).

Fig. 2.7b. Detail of Fig. 2.7a. (Bar = 25 μm).

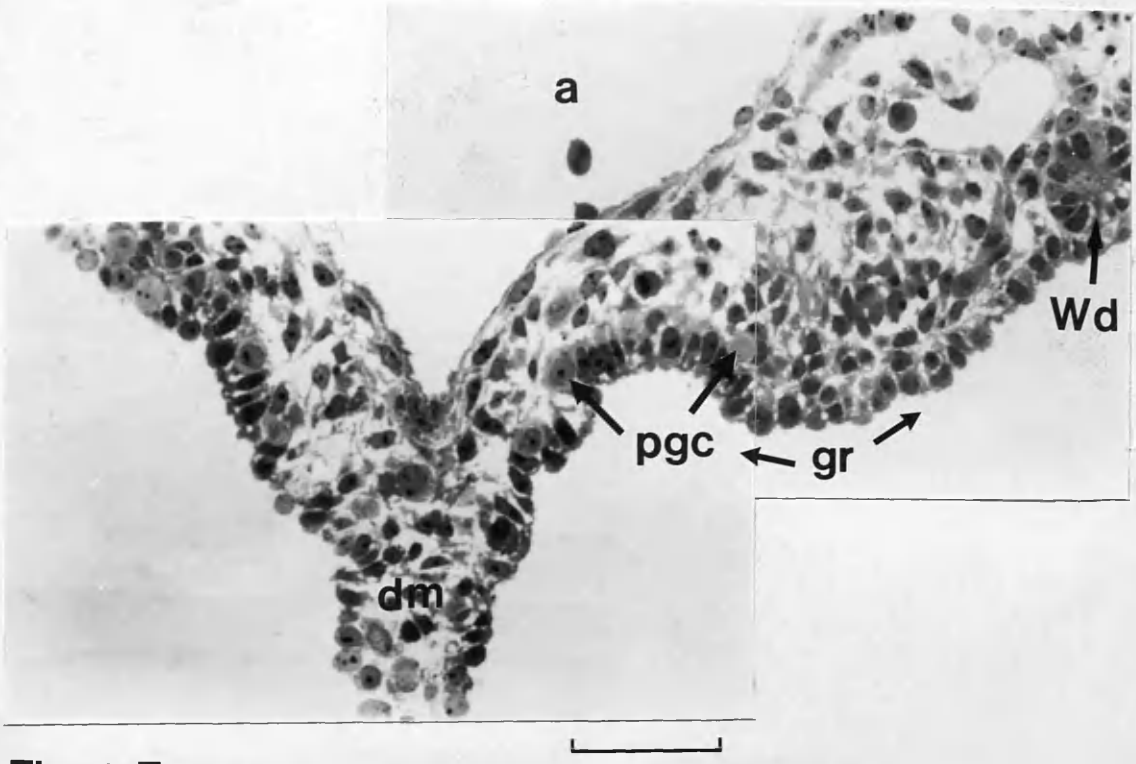


Fig. 2.7a

Fig. 2.7b

Fig. 2.8a. Transverse section through the gonadal ridge and the mesonephros of a 10.5 day old embryo. (Bar = 100 μm).

Fig. 2.8b. Detail of Fig. 2.8a. Note the presence of lysomsome-like bodies (llg) in the mesonephric tubule cells (mt). (Bar = 25 μm).

Fig. 2.9. Transverse section through the gonadal ridge of a 10.5 day old mouse embryo. Note the mitotic figure (mf) in the mesothelium (m) and the blood capillary (bc) with nucleated erythrocytes. (Bar = 50 μm).

Fig. 2.10. Electron micrograph through the gonadal ridge of a 10.5 day old mouse embryo. Note the lack of distinction between the superficial and the underlying cells. x 2.4 K.

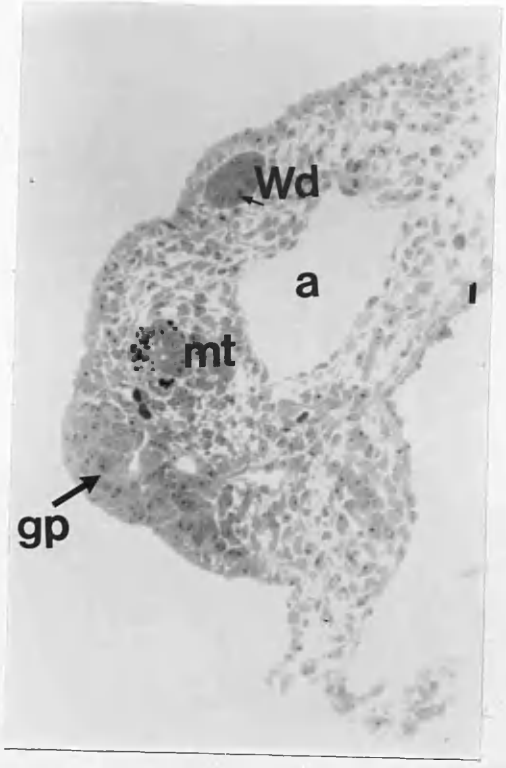


Fig. 2.8a

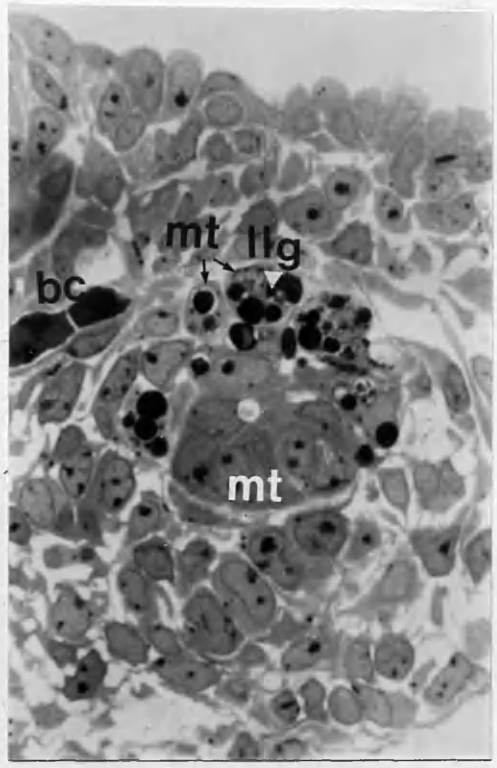


Fig. 2.8b

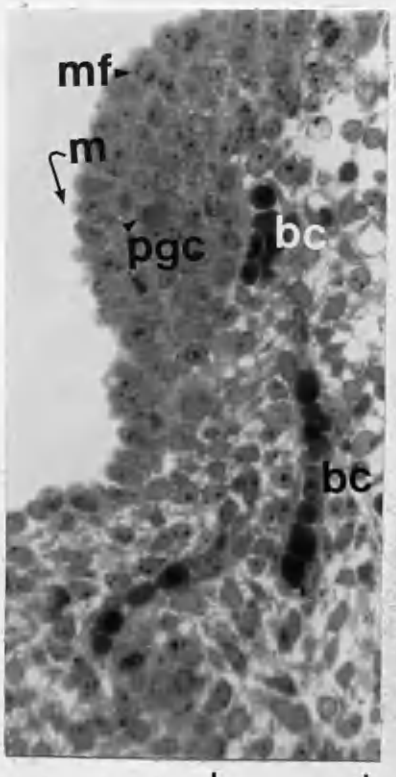


Fig. 2.9

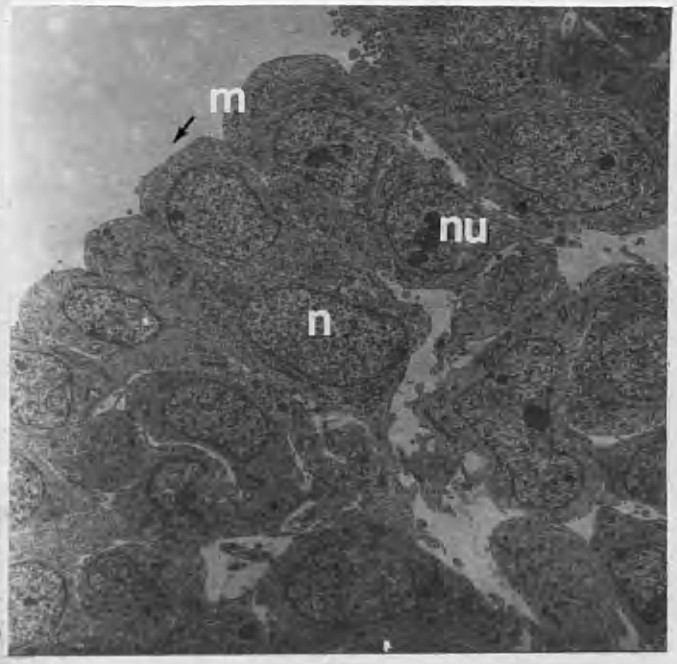


Fig. 2.10

Fig. 2.11a. Transverse section through the gonadal ridge and the mesonephros of an 11 day old mouse embryo. Note irregular profile of mesonephric cords (msc). (Bar = 100 μm).

Fig. 2.11b. Detail of Fig. 2.11a in the region of the gonadal ridge (gr). (Bar = 25 μm).

Fig. 2.12. Electron micrograph through the gonadal primordium of an 11 day old mouse embryo. x 1.5 K.

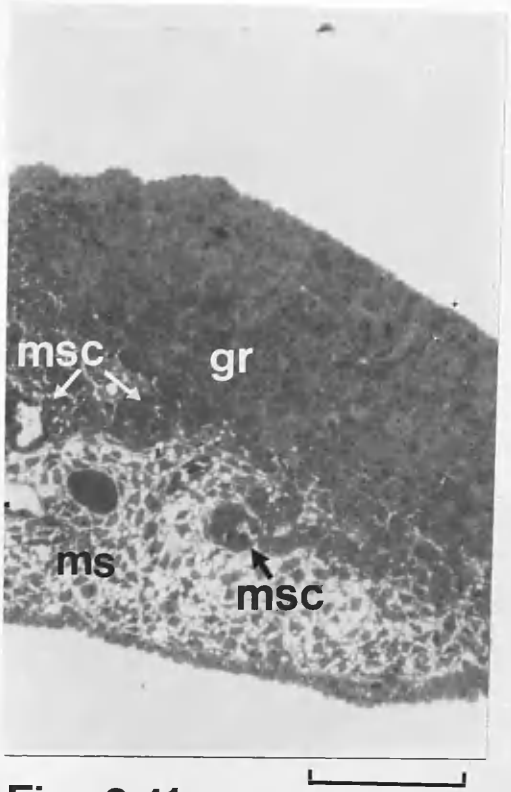


Fig. 2.11a

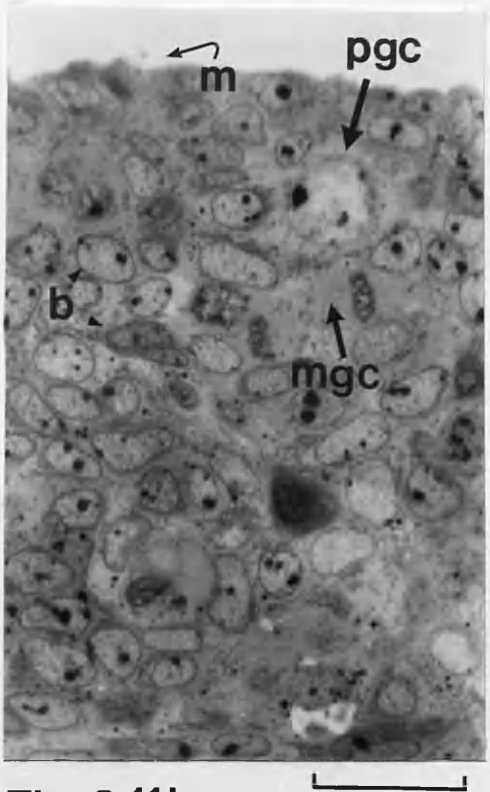


Fig. 2.11b

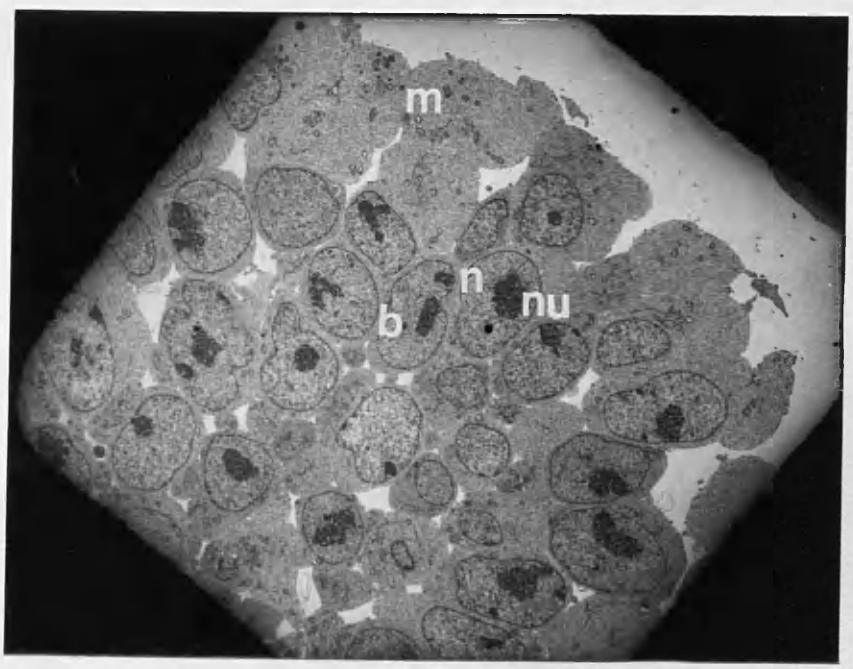


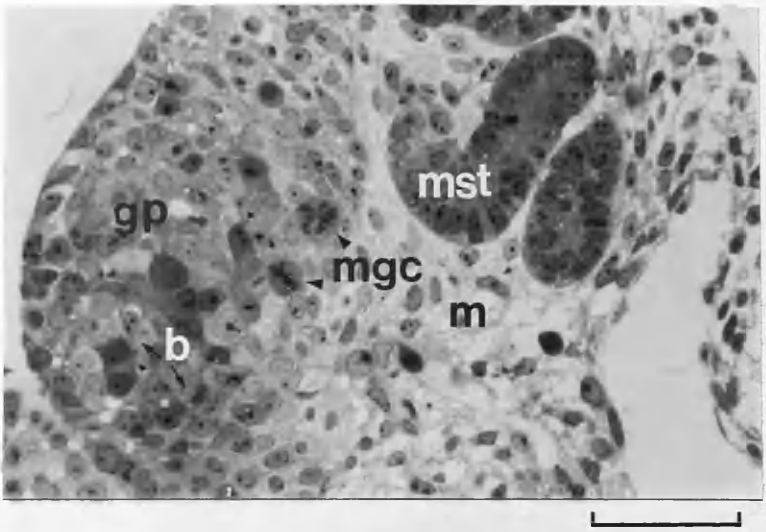
Fig. 2.12

Fig. 2.13. Transverse section through the gonadal primordium (gp) and the mesonephros (ms) of an 11 day old embryo showing an S-shaped mesonephric tubule (mst). (Bar = 50 μm).

Fig. 2.14a. Electron micrograph through an S-shaped mesonephric tubule (mst) of an 11 day old mouse embryo. x 1.28 K.

Fig. 2.14b. Detail of Fig. 2.14a. Note the absence of a basal lamina around the mesonephric tubule (mst). x 2.5 K.

Fig. 2.13



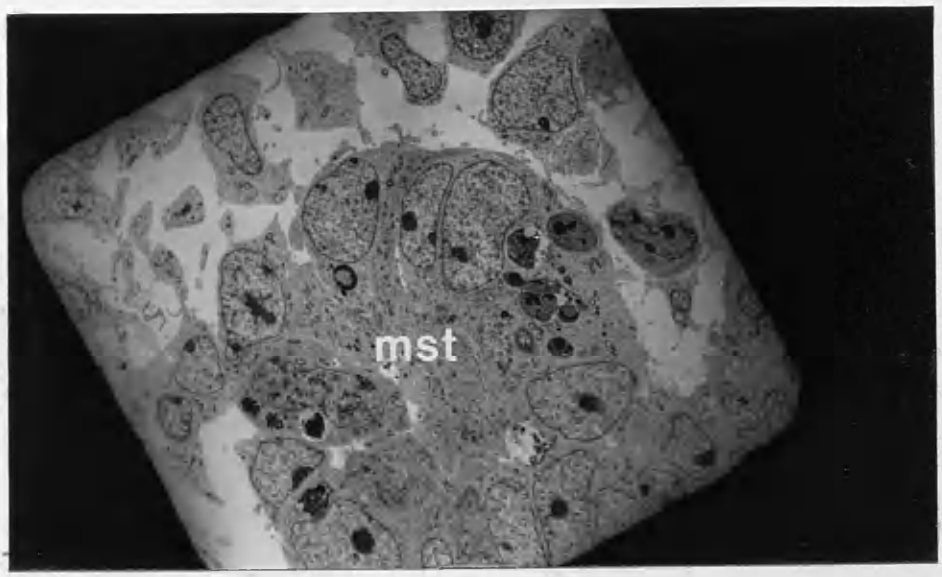


Fig. 2.14 a

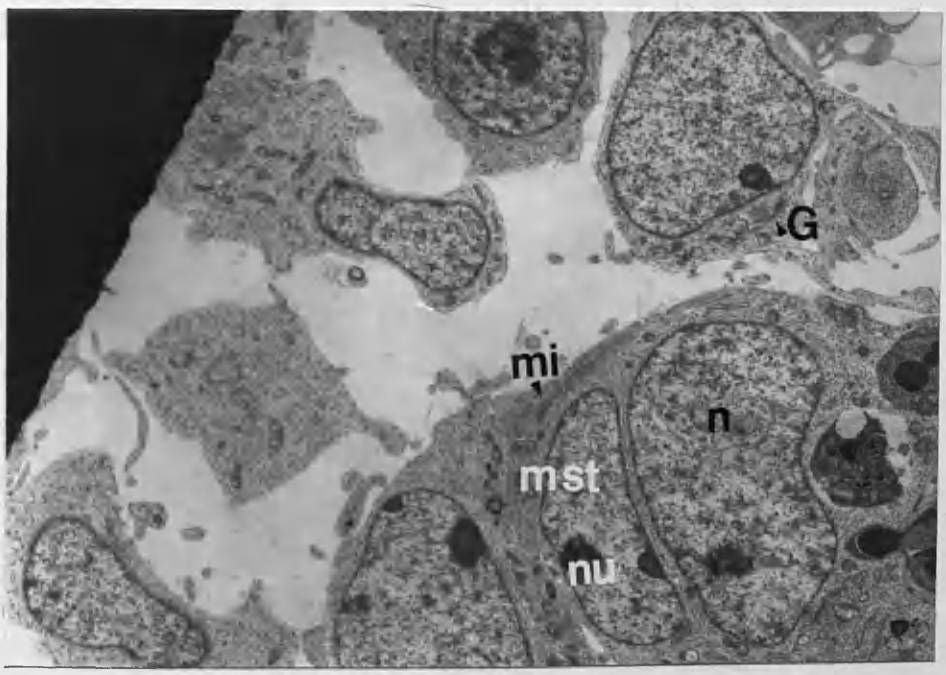


Fig. 2.14 b

Fig. 2.15. Transverse section through the gonad of a genetic female at day 11.5 of gestation. Note the compact structure of the putative ovary and the nucleated erythrocytes (e). (Bar = 50 μm).

Fig. 2.16a. Transverse section through the gonad of a genetic male at day 11.5 of gestation. (Bar = 50 μm).

Fig. 2.16b. Detail of Fig. 2.16a to show the presumptive tunica albuginea (ta) and a blood capillary (bc). (Bar = 25 μm).

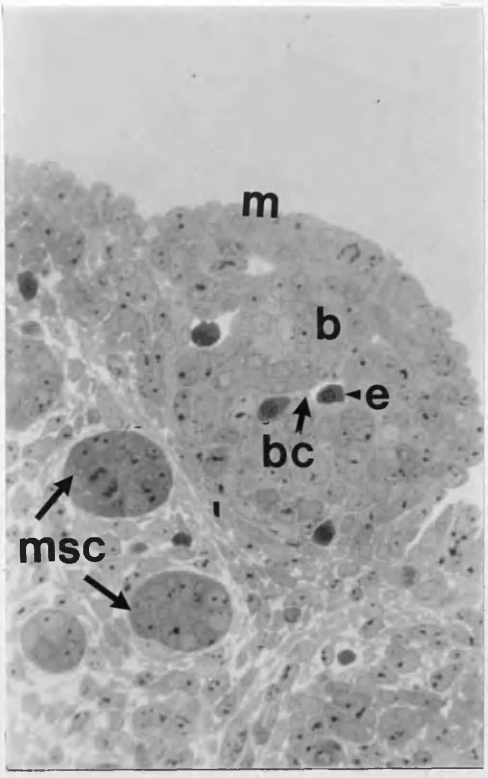


Fig. 2.15

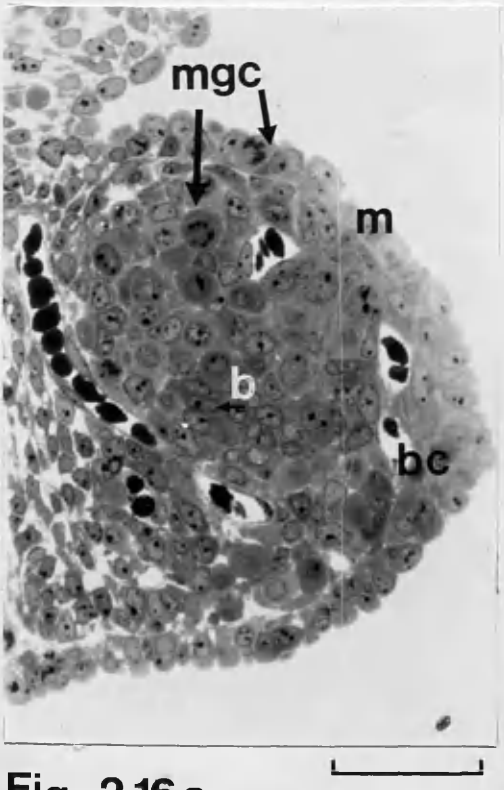


Fig. 2.16a

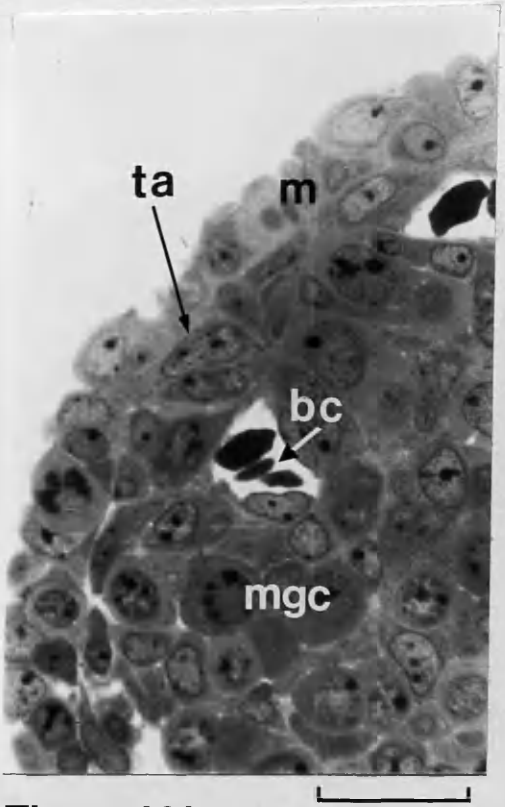


Fig. 2.16b

Fig. 2.17a. Transverse section through the testicular rudiment (tr) and the mesonephros (ms) of a 12 day old mouse embryo. (Bar = 100 μm).

Fig. 2.17b. Detail of Fig. 2.17a. (Bar = 25 μm).

Fig. 2.18a. Transverse section through the ovarian rudiment (or) and the mesonephros (ms) of a 12 day old embryo. (Bar = 100 μm).

Fig. 2.18b. Detail of Fig. 2.18a, showing a mesonephric tubule with irregular profile (mst). (Bar = 25 μm).

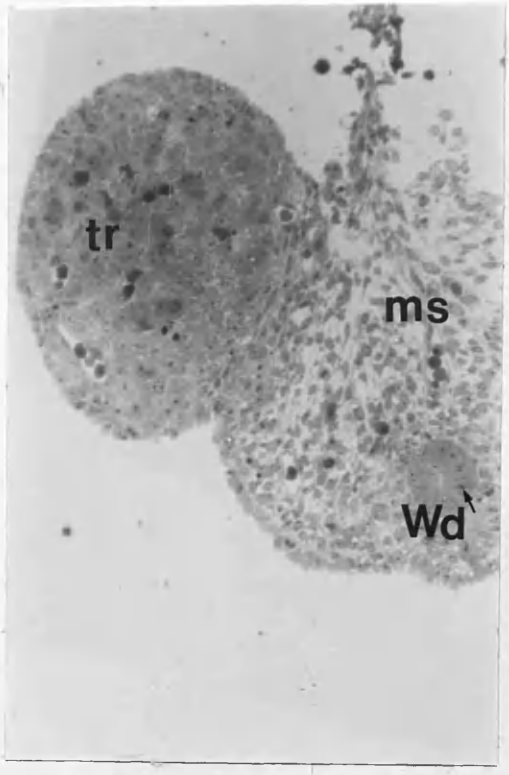


Fig. 2.17a

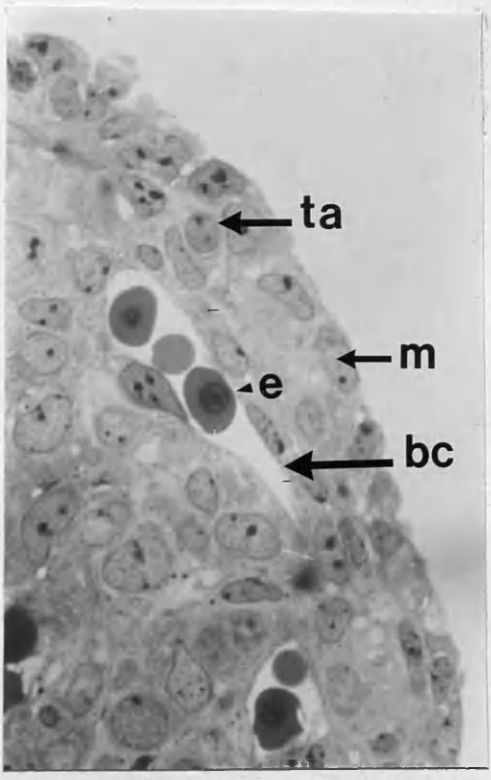


Fig. 2.17b

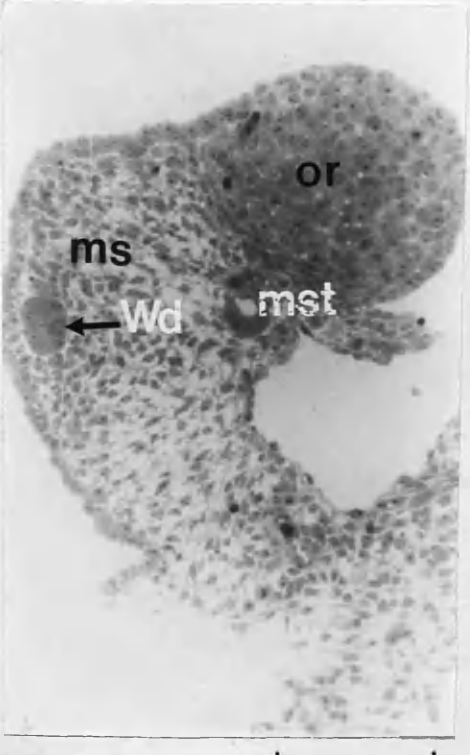


Fig. 2.18a

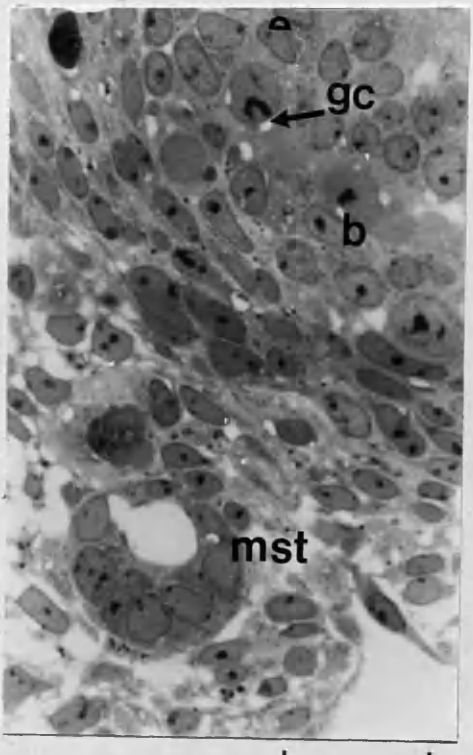


Fig. 2.18b

Fig. 2.19a. Longitudinal section through the testicular rudiment at day 13 of gestation. (Bar = 50 μm).

Fig. 2.19b. Detail of Fig. 2.19a, showing the primordial germ cells (pgc) and the pre-Sertoli cells (pS) clumping together forming sex cords (sc), and mitotic germ cell (mgc) embedded in the mesothelium (m). (Bar = 25 μm).

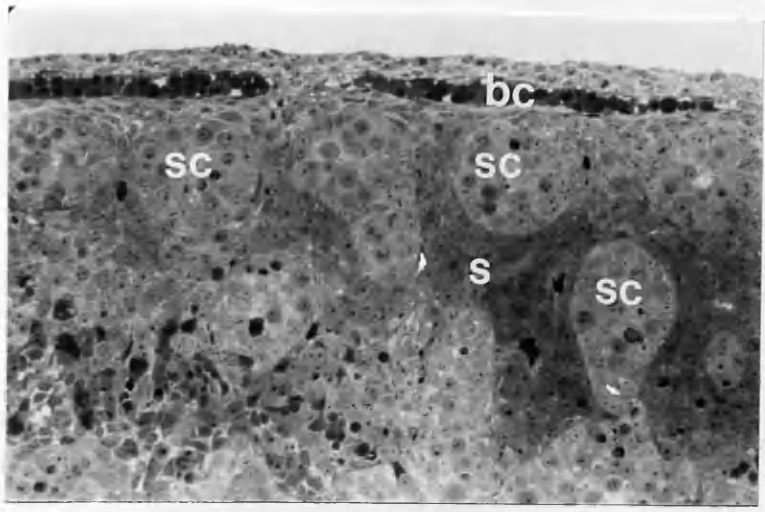


Fig. 2.19 a

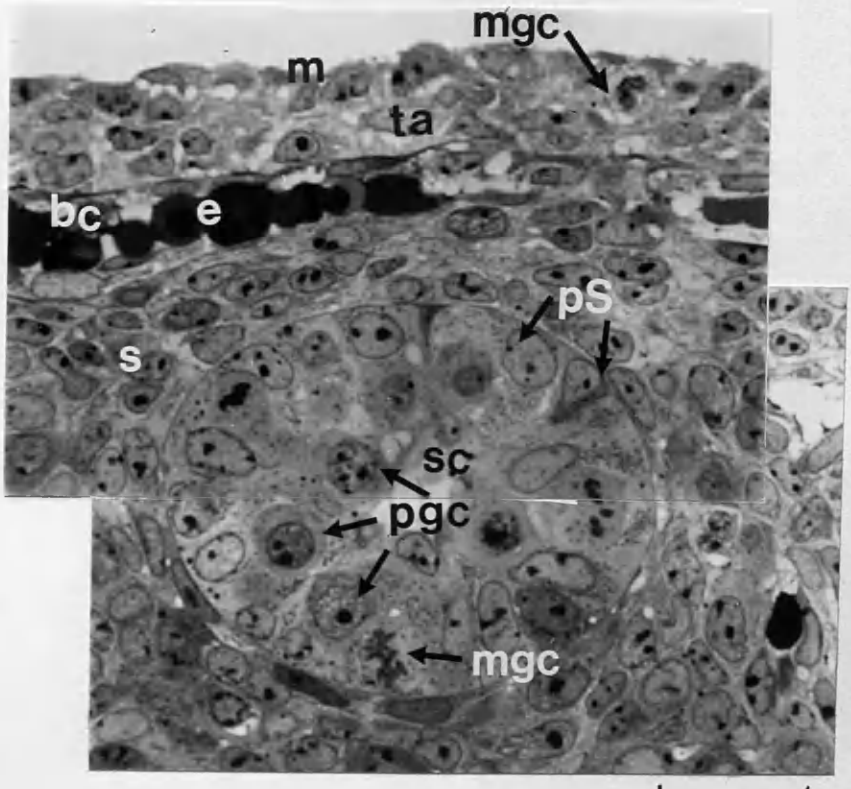


Fig. 2.19 b

Fig. 2.20. Transverse section through the ovarian rudiment at day 13 of gestation. Note the dividing germ cells (mgc) to form oogonia and the irregular mesothelium (m). (Bar = 50 μm).

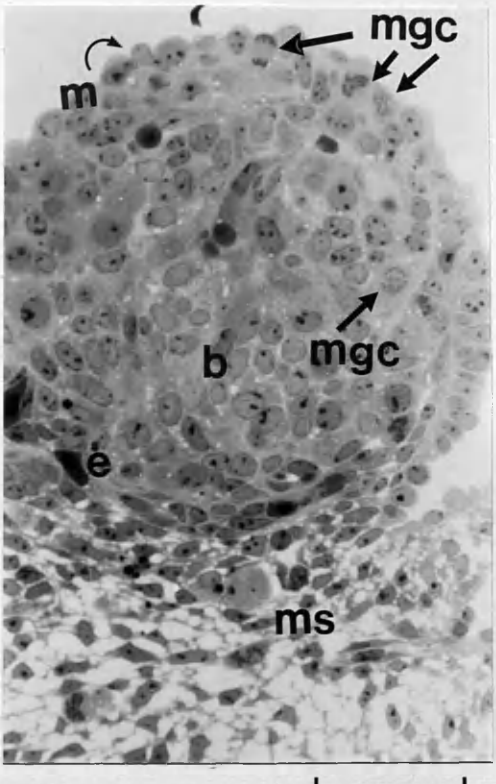


Fig. 2.20

Fig. 2.21a. Transverse section of a portion of the testicular rudiment at day 14 of gestation. Note the regular mesothelium (m) and the well developed tunica albuginea (ta). (Bar = 50 μm).

Fig. 2.21b. Same as 2.21a at the hilar region , showing the relation between a mesonephric tubule (mst) and the testicular rudiment (tr). (Bar = 50 μm).

Fig. 2.22a. Portion of an ovarian rudiment at day 14 of gestation, showing oocytes at the leptotene (ol) stage of the first meiotic division. (Bar = 25 μm).

Fig. 2.22b. Same age embryo as in Fig. 2.22a, but oocytes at the zygotene stage (oz) of the first meiotic division. (Bar = 25 μm).

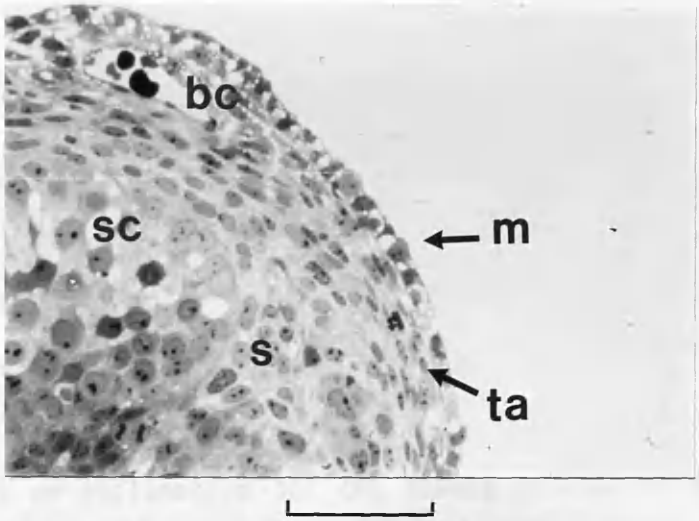


Fig. 2.21a

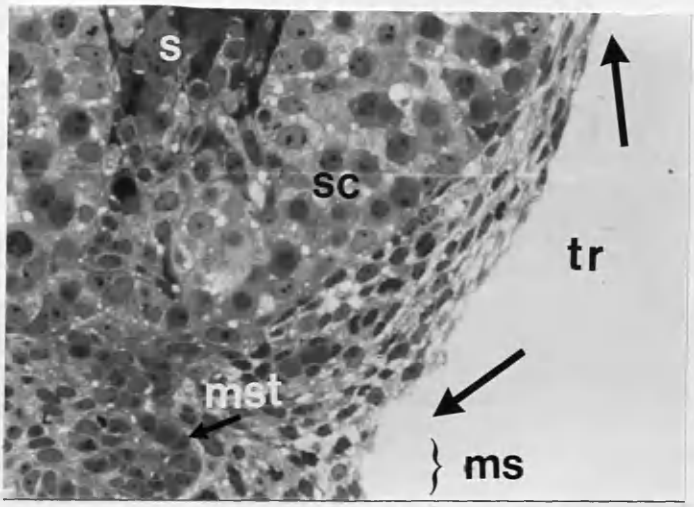


Fig. 2.21b

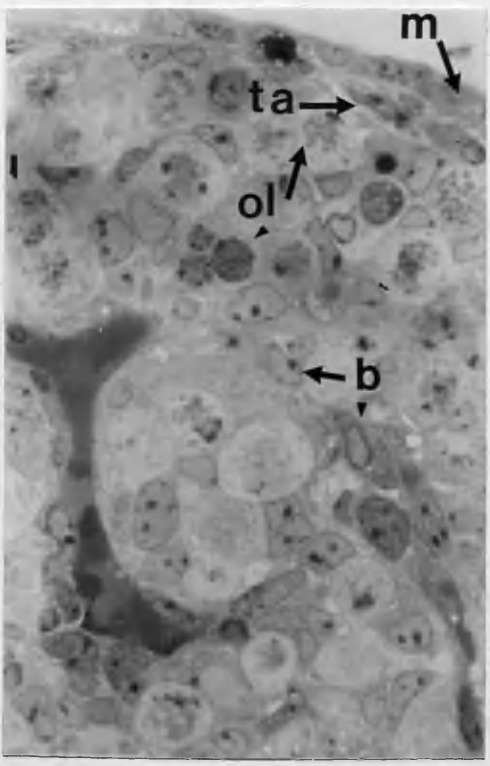


Fig. 2.22a

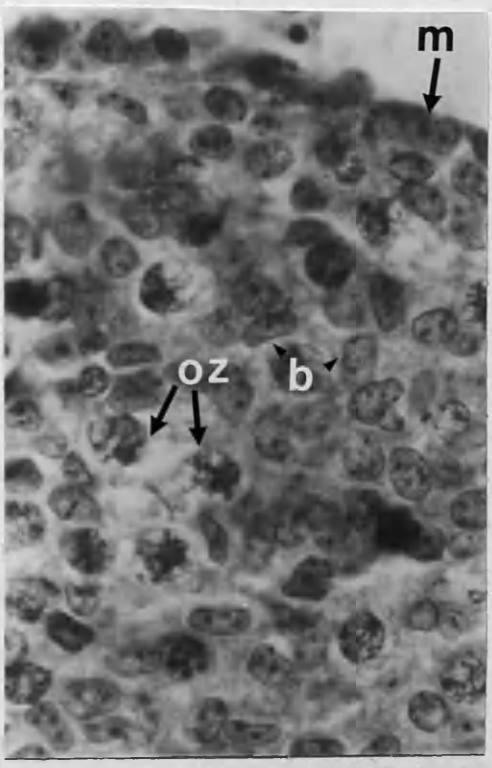


Fig. 2.22b

2.4. DISCUSSION

Witschi (1948) working on human embryos postulates that the PGCs reach their final destination under the influence of a chemical substance released from the mesothelial cells of the gonadal ridges. The observations presented here show that the PGCs arrive at the location of the future gonadal ridges before the latter develop. Therefore such an explanation for the homing of PGCs is difficult to accept, at least in the mouse.

Authors differ as to the time when the gonadal ridges first appear. While Brambell (1927) recognizes the gonadal ridges on day 8 *post coitum*, Upadhyay *et al* (1979) state that they form on day 11 of fetal life. The present observations indicate that the gonadal rudiments develop on day 9 of gestation. The variation in the time of first appearance of the gonadal ridges may be due to species differences. However, Upadhyay *et al* (1979) did not study the development of the gonad primordia before day 11 of gestation.

The structure of gonadal ridges of male and female embryos of known chromosomal sex has been studied in the pig (Pelliniemi, 1975a &b) and human (Pelliniemi, 1976). No study is available describing the early stages of gonadal development in mouse embryos of known chromosomal sex. Pelliniemi (1975a &b) observed that there was no difference between the structure of the genetically male and female pig gonads at day 24 of fetal life. He states that the testis can be distinguished by the presence of seminiferous cords by day 26 of gestation.

The observations presented in this study indicate that there are no differences in the structure of genetically male and female mouse embryos at day 9-11 of gestation. At day 9 *post coitum*, the mesothelium in both sexes consists of a layer of columnar cells which

lack a basal lamina. The mesothelial cells are connected to each other by junctional complexes such as tight junctions and desmosomes. The mitochondria are elongated or spherical with irregularly arranged cristae and may be associated with rough endoplasmic reticulum. At day 10 of gestation, the mesothelium becomes irregular in thickness. The mesonephric cords have irregular profiles, lack a basal lamina and contribute cells to the gonadal blastema in both sexes. By day 11.5 post coitum, however, a difference exists between the genetically male and female gonad rudiments. The former appear larger in sections and a capillary penetrates the periphery. The observations are thus in agreement with Pelliniemi (1975a &b, 1976) in that no differences are present between the genetically male and female gonadal rudiments at early stages of development. In this study, however, the first obvious difference noticed was the appearance of the tunica albuginea and not the seminiferous cords as is reported by Pelliniemi (1975) for the pig.

As far as the mouse is concerned, three theories have been proposed as to the origin of the blastema. Firstly, the blastema arises from the mesothelium of the gonadal ridges (Kingery, 1919; Brambell, 1927). According to these authors the proliferation of the mesothelium occurs in the form of irregular masses and not in the form of cords or tubules. The second theory postulates that the blastema has a mesonephric origin (Upadhyay et al., 1979, 1981). These investigators state that the mesonephroi have rudimentary organization and lack conventional glomeruli and therefore are incapable of any excretory activity. They found that at later stages of development (that is at day 11 of gestation) they become organized into mesonephric tubules. From the ventral extremeties of the latter, mesonephric cells originate and then begin to colonize the gonadal

area.

Upadhyay et al. (1979) report that mesonephric cells which are about to or in the process of being mobilized from the mesonephric tubules are characterized by the presence of lysosome-like bodies. They suggest that these organelles are probably used for the enzymatic digestion of the basal lamina of the mesonephric tubules.

A dual origin of the blastema, from mesothelial and mesonephric cells, is a third idea, proposed by Wartenberg (1981, 1983). In his review, Wartenberg (1981) states that the mesonephric rudiments segregate cells at the time when they are still made up of undifferentiated masses of nephrogenic cells (that is at day 9 post coitum) and later on when they become organized into S-shaped mesonephric tubules (that is at day 11 of gestation). He states that the ventral extremities of the latter differentiate into small vesicles from which cells are detached. Fraedrich, 1979 (quoted in Wartenberg, 1981) also found that some of the mesonephric cells which are detached from mesonephric tubules have dark lysosomal granules and similar cells can be traced into the gonadal ridges.

The present observations are in agreement with Wartenberg (1981, 1983) regarding the dual origin of the blastema, from both the mesothelial and mesonephric tubule cells. The observations are also similar to those reported in this study for the tammar wallaby (Chapter 3, p. 141).

At the earliest stage studied (that is at day 9 post coitum), it was found that whereas the mesothelium elsewhere is flattened, in the region of the presumptive gonadal ridges it consists of a layer of highly columnar cells which lack a continuous basal lamina. The mesonephric rudiments at this time consist of undifferentiated nephrogenic masses having irregular profiles. At later stages of development (that is in the indifferent stages, day 10 and 11 of

gestation), the mesothelium exhibits many mitotic figures, the spindles of which are at right angles to the gonadal surface. In the absence of the basal lamina, there is no line of demarcation between the mesothelium and the underlying blastema cells. At this stage of development the former is regular, consisting of columnar and cuboidal cells which appear as pseudostratified epithelium in some regions. During the indifferent stage, cells which are found between the gonadal rudiment and the mesonephric tubules are thought to derive from the latter because of the irregular profile of the mesonephric tubules and the lack of a continuous basal lamina.

In this study mesonephric cells with darkly staining granules, such as are described by Upadhyay et al (1979) and Wartenberg (1981), were also found among the cells of the mesonephric tubules: these might be required for enzymatic digestion of the basal lamina, as suggested by the former authors.

Wartenberg (1983) postulates that an interaction between the mesonephric cells and both the mesothelial cells and the PGCs occurs. According to him, this interaction results in the proliferation of the mesothelial cells and stimulation of PGC migration to the gonadal ridges. He suggests that the mesonephric cells promote the migration of PGCs by cellular contact using long thin cytoplasmic processes.

The observations presented here agree with Wartenberg (1981) regarding the time of development of the rudimentary mesonephroi but they differ from his interpretation in the time of the first appearance of putative gonadal ridges. The proliferation of the mesothelial cells and the arrival of PGCs to the future gonadal ridges occur 2 days earlier than the time suggested by Fraedrich,1979 (quoted in Wartenberg, 1981). In this work, it was found that mesothelial proliferation and PGC attraction to the presumptive gonadal ridges do

not occur under the influence of mesonephric cell migrations to the gonadal ridges as stated by Wartenberg. If the mesonephric cells had a stimulatory effect on the proliferation of the mesothelium and attracted PGCs to the gonadal ridges, one would expect the mesonephric cells to be formed at earlier stages of development than the gonadal ridges.

Wartenberg (1983) suggests a dual function for the mesothelial and the mesonephric cells which, according to him, constitute the blastema. He states that the mesonephric and the mesothelial cells have stimulatory and inhibitory effects respectively on the mitotic activities of the PGCs.

The observations in this study contrast with the idea of Wartenberg (1983) regarding the stimulatory effect of the mesonephric cells on the PGCs, as the latter are found to be in mitosis at very early stages of development, that is, when they are still in the hindgut and when they are embedded in the putative gonadal ridges. In other words, they are dividing long before reaching the gonadal ridges and interact with the mesonephric cells as is suggested by Wartenberg (1983).

The observations presented here are also in disagreement with Wartenberg's idea of the inhibitory effect of the mesothelium on the mitotic activities of the PGCs. If the mesothelium had an inhibitory effect on PGCs, one would not expect to find numerous dividing PGCs among the mesothelial cells. On the contrary, mitotic PGCs are found abutting on the mesothelium at almost all stages of gonadal development studied.

Although the reproductive capacities of the gonads establish only after puberty, their sex can be recognized early in fetal life (Byskov, 1981). The indifferent gonads become sexually recognizable when the testes start to differentiate in genetic males (Jost, 1970).

At this time, the presumptive females do not show any characteristic ovarian structure (Brambell, 1927; Franchi, 1962; Jost, 1970; Byskov, 1981; Ullmann, 1981a; Wartenberg, 1981). They continue to grow for some time and then they differentiate.

There are various ideas in the literature regarding the morphological aspects of gonadal sexual differentiation (see Section 1.3). As far as the mouse is concerned, three views have been expressed. Some authors believe in mesonephric induction of sexual differentiation (Brambell, 1927; Upadhyay, 1979, 1981). These authors report that the first indication of testes development is the appearance, at the periphery of the gonads, of the putative tunica albuginea. The latter develops as a downgrowth of either mesenchymal (Brambell, 1927) or mesonephric (Upadhyay, 1981) cells from the base of the gonadal rudiments, between the mesothelium and the gonadal blastema.

The second view postulates that an "autodifferentiation" of the blastema cells results in testis differentiation (Jost, 1981). This author studied testis development in rat embryos. However, according to him, the same pattern of sexual differentiation is applicable to the mouse. This supposition is supported by the work of Byskov (1981) on the mouse: they observed that testicular cords developed in fetal indifferent mouse gonads cultured without the attached mesonephroi. Accordingly, these investigators suggest that the trigger for testicular differentiation is present within the testicular primordia themselves. They suggest that this trigger for seminiferous cord development, which is postulated by Wachtel et al (1975) to be an H-Y antigen, must be expressed just before the differentiation of the testis. However, this antigen is not only confined to males, but is present in the female of birds and Xenopus, the African clawed toad

(Wachtel et al., 1975; Mittwoch, 1977). Moreover, McLaren et al (1984) found that H-Y antigen was absent from certain mice of male phenotype and therefore suggest that it is unlikely to be responsible for testis determination.

Wartenberg (1981) has suggested that the morphological aspects which lead to sexual differentiation of the gonads depend on the interaction between the gonadal blastema cells, which he believes have a dual origin, from both the mesothelial and the mesonephric cells. He considers his view, which is based on comparative studies of gonads in different mammalian species, to be similar to Witschi's (1956) concept of cortico-medullary antagonism (see Section 1.3). However he states that sexual differentiation initiates as a result of interaction between the antagonistic elements of the gonadal blastema rather than the dominance of one compartment (either the cortex or the medulla) over the other, as was proposed by Witschi. According to Wartenberg (1981), the distribution of the two kinds of cells proceeds differently during differentiation of the two sexes. Wartenberg's (1983) account, however, is ambiguous: on the one hand he states that the blastema is a mixture of mesonephric and mesothelial cells, while on the other hand, he reports that dark (mesonephric) cells penetrate the gonadal blastema. He states "In the case of male differentiation the tendency of the dark, mesonephric cells to penetrate the gonadal blastema in a peripheral direction, increases until the dark cells dominate in the superficial region. If a testis develops, a layer of dark cells finally separates the gonadal blastema from the most superficial epithelium. This layer represents the primordium of the tunica albuginea ... In the case of female differentiation, dark cells hardly penetrate into the superficial region."

In this study it was found that on day 12 of gestation some of the blastema cells swell, acquire less affinity for the stains used

and clump together enclosing the prespermatogonia to form the sex cords. Thus the observations here are consistent with those of Jost (1981) and those reported in this study for the tammar wallaby, the bettong and the potoroo (Chapter 3, p. 144). Sexual differentiation of the testis occurs as a result of "autodifferentiation" of the blastema forming the seminiferous cords. The results are also in agreement with Byskov et al (1981) in that the trigger for testis differentiation is present within the testicular rudiments themselves.

While most investigators believe that the appearance of the tunica albuginea is the first indication of testicular differentiation (Brambell, 1927; Upadhyay et al., 1979), some report that the first sign of incipient testicular development is the aggregation of PGCs and their enclosure by the future Sertoli cells to form the testicular cords (Byskov, 1981).

The results reported here reveal that the sexual differentiation of the indifferent gonad into a testis is initiated on day 12 post coitum. The first indication of testis development is the appearance of a line of demarcation between the investing mesothelium, which becomes regular in thickness, and the underlying blastema. At this time connective tissue cells (the putative tunica albuginea) appear in the future testis between the mesothelium and the gonadal blastema. Simultaneously with the development of the tunica albuginea, blood capillaries invade the periphery of the testis and extend along its length.

The development of the presumptive seminiferous cords is the second event of testicular differentiation observed.

The observations presented here are in agreement with those described by Brambell (1927) and Upadhyay (1979) in that the first event of sexual differentiation of the testis in the mouse is the

development of the presumptive tunica albuginea and not the seminiferous cords as is reported by some authors (Byskov, 1981).

The present observations confirm the well known fact that during the period of sexual differentiation of the testis, the prospective ovary maintains the features of the indifferent stages described above. It is made up of randomly distributed germinal and blastemal cells and invested with a mesothelium which is irregular in thickness. At this time, the mesothelium of the putative testis becomes regular in thickness.

The results presented here indicate that at the indifferent stage and in the ovarian rudiments, both the mesothelial and the blastemal cells vary in their affinity for the stains used. At the time of sexual differentiation of the gonads, some of the blastema cells differentiate into connective tissue cells (the tunica albuginea) while others become pre-Sertoli cells. Wartenberg (1983) believes that sexual differentiation of the testis in the rabbit and mouse initiates as a result of dissimilar organization of the mesothelial and the mesonephric cells to which he imputes different staining characteristics (see p. 64). The results of this study do not support such an interpretation and it appears that these ideas are not applicable to the mouse, since neither the size, shape nor affinity for the stains are reliable criteria for distinguishing between these 2 cell types at early stages of gonad development.

The development of mouse ovaries has been the subject of several studies (Brambell, 1927; Borum, 1969; Upadhyay et al., 1979; Byskov, 1981; Grinsted, 1981; Fraedrich, 1979, quoted in Wartenberg, 1981). Mouse ovaries are characterised by immediate meiosis: oogonia enter the prophase of the first meiotic division 1 day (Brambell, 1927; Borum, 1961), 2 days (Upadhyay et al., 1979) or 3 days (Odor & Blandau, 1969) after gonadal sexual differentiation. The observations

in this study indicate that oogonia enter meiotic division a day after gonadal sexual differentiations (see p. 43). Therefore the results are consistent with those of Brambell (1927) and Borum (1961). The variation between different authors in the time of initiation of meiosis may be due to species differences since different species were used.

While most investigators (Brambell, 1927; Odor & Blandau, 1969; Upadhyay et al., 1979) do not refer to the synchronization of the rmeiotic division of oocytes, Borum (1961) states that the synchronism is absolute only after 2 days of the initiation of meiosis. She found that oogonia, leptotene and zygotene stages are all present on day 14 post coitum while pachytene is the dominant stage in the ovaries of 16 day old embryos. The results presented here (p.43) show that on day 13 of gestation only some of the oogonia enter the prophase of the first meiotic division but by day 14 the majority have entered meiosis and are found in groups of morphologically similar cells. The results are thus in agreement with those of Borum (1961) in that the synchrony of the divisions is not complete when meiosis first begins. The observations, however, differ from those of Borum in that synchrony in divisions occurs 1 day and not 2 days after the commencement of meiosis. The results also differ from those described in this study for the potoroo, in which the meiotic activities of the oocytes are not synchronized (Chapter 3, p. 157).

In this study pupoid foetus which is a mutant species of mice has been used. The homozygous pupoid foetus embryos show some characteristic ectodermal abnormalities due to the presence of mutant genes and the mutation is situated upon chromsome no. 8 (Watson, 1978).

The first external morphological features which distinguish

mutant from normal embryos are the presence of a small distinct tail twist and rounded forelimbs. On the other hand, in the similarly aged normal embryos, the tail is quite straight and the forelimbs tend to be paddle shaped and show the first sign of foot plate development. These peculiarities do not express themselves until late in development, that is at day 11.5 of gestation.

Since the mutation affects ectodermal structures it is perhaps not surprising that no abnormalities were detected in the gonad stages studied.

CHAPTER THREE

GONAD DEVELOPMENT IN MACROPOD MARSUPIALS: *MACROPUS EUGENII*, *BETTONGIA GAIMARDI* AND *POTOROUS TRIDACTYLUS*

3.1. INTRODUCTION

The development of gonads from the indifferent stages has been described for 5 species of marsupials (opossum: Moore, 1939; Morgan, 1943; native cat: Fraser, 1919; Ullmann, 1984; long-nosed and short-nosed bandicoots: Ullmann, 1981b; tammar wallaby: Alcorn, 1975). O'Donoghue (1912) reported on the corpus luteum and polyovular follicles (POFs) in the native cat and on the interstitial gland tissue (IT) in the brush-tailed possum (O'Donoghue, 1916). Hartman (1926) described POFs in the ovaries of the opossum.

Moore (1939) and Morgan (1943) maintain the now obsolete idea of the mesothelial origin of primordial germ cells (PGCs).

In marsupials the follicle cells have been described as arising from the stroma (Morgan, 1943), the rete cords (Alcorn, 1975) or from both the medullary cords and the mesothelium (Ullmann, 1986).

While the IT has been considered to differentiate either from the stroma (O'Donoghue, 1918) or from undifferentiated cells in the stroma (Ullmann, 1984b), Alcorn (1975) believes that the IT originates from the medullary cords.

POFs have been reported in the ovaries of the native cat (O'Donoghue, 1912), opossum (Hartman, 1926); tammar wallaby (Alcorn, 1975) and potoroo (Ullmann, 1983). O'Donoghue (1912) and Hartman (1926) found that POFs form as a result of insufficient numbers of follicle cells. On the other hand, Alcorn (1975) believes that POFs originate from a failure of oogonia, which are joined by intercellular bridges, to separate before they become surrounded by follicle cells.

Most of the studies on marsupial gonads are at the light microscopic level. The only ultrastructural observations available are those of Alcorn (1975) and Ullmann (1981b) on the PGCs of the tammar and bandicoot respectively; and those of Ullmann (1978) on the primary

follicles of the bandicoot.

This chapter attempts to provide a description of gonad development in macropod marsupials utilizing 3 different species, Macropus eugenii, Bettongia gaimardi and Potorous tridactylus. The study was mainly performed at the light microscopic level but where material was available (as in the case of the potoroo) also at the ultrastructural level.

In the potoroo, the study also includes some quantitative analysis of ovarian growth parameters, i.e. estimation of both ovarian volumes and altered cortical and medullary volumes with age.

Although gonad development in the tammar wallaby has previously been investigated by Alcorn (1975), embryonic gonads are reinvestigated in this study to provide the indifferent stages, which are not available for the bettong or the potoroo. The latter are slow breeders (Bates et al., 1972) and it is difficult to obtain the appropriate stages.

Other aims of this project are to try and throw some light on the hitherto unsolved problems regarding the origin of the different tissues of the gonad which have been listed in Section 1.1 and to compare the process of gonadal differentiation in marsupials and eutherians.

3.1.2. Description and distribution of the macropods used in this study: Potorous tridactylus, Bettongia gaimardi and Macropus eugenii

3.1.2.1. Potorous tridactylus

The potoroo is one of the lesser known Australian marsupials (Guiler, 1960) because it is nocturnal (Bates et al., 1972; Collins, 1973). It is now rare in mainland Australia, but still common in Tasmania where it is fully protected (Guiler, 1960; Hughes, 1962;

Bryant & Rose, 1986). Its natural habitat is among densely growing grasses or heaths particularly in wet areas (Bates et al., 1972).

The form of the body is typically macropod (i.e. like the kangaroo, see Fig.3.2) but the hind legs do not reach the same development as in the kangaroo. The tail is rat-like, and that is why it is also called the rat kangaroo (Guiler, 1960). The size of the adult potoroo is about the same as that of a rabbit and its weight is about 1500gm (Ullmann & Brown, 1983).

The potoroo is polyoestrous and breeds throughout the year (Hughes, 1962; Bryant & Rose, 1986). It has the longest gestation length (about 38 days) known in marsupials and one of the longest oestrous cycles (42 days) within the Macropodidae (Rose, 1978). The potoroo is monovular and has a very low reproductive potential (Bates et al., 1978).

The early development of the young takes place inside the uterus. The placenta is primitive, develops from the yolk sac and is called a yolk sac placenta.

The young is born in a very immature state of development inspite of the relatively long gestation period. It continues its development in a pouch or marsupium which contains the teats of the milk glands. At birth most of the sense organs are non-functional and the eyes are not open (see Fig. 3.3). The hindlimbs of the potoroo pouch young are smaller than the forelimbs at birth, but gradually become larger and assume the macropod form. The potoroo has a relatively short pouch life of 3-4 months (Rose, personal communication); the young suckle for about 2 months while permanently attached to the teat and for another 2 months after which they vacate the pouch (Ullmann & Brown, 1983).

3.1.2.2. Bettongia gaimardi

The bettong is a rat kangaroo characterised by its burrowing habit (Tyndale Biscoe, 1969). Previously it was distributed on mainland Australia but now it is found only in the open forest habitats of eastern Tasmania (Rose, 1986). The latter considers the conservation state of the bettong to be in danger due to its liability to the introduction of eutherian predators and changes in land use. Like the potoroo, the bettong is a continuous breeder and displays embryonic diapause. That is, the females enter post partum oestrous and the resulting embryos develop to an early blastocysts stage but then remain quiescent until the pouch young is lost or stops suckling (Shaw & Rose, 1979).

In contrast to the potoroo, the bettong has the shortest gestation length (21.1 days) and oestrous cycle length (23.2 days) of the macropods (Rose, 1978). The pouch life is approximately 3-4 months.

3.1.2.3. Macropus eugenii (Fig. 3.4)

The tammar is a small wallaby which lives in low scrub where it moves rapidly, hopping low to the ground with the forelimbs held away from the body (Alcorn, 1975). It is distributed in south and west Australia but is common now in the two islands of Houtman's Abrohos and on Kangaroo islands (Alcorn, 1975).

The tammar is a seasonal breeder (Tyndale-Biscoe, 1968; Renfree, 1982) and has a gestation length of 28.3 days and an oestrous cycle of 28.4 days (Rose, 1978).

3.2. MATERIALS AND METHODS

3.2.1. Source of materials

3.2.1a. Bettongia gaimardi

13 female pouch young, ranging in age between 2.5 days and 12

weeks were used (see Appendix). They were obtained, in a fixed state, from two sources:

1) Dr D. Darrell Kitchener, Curator, Western Australian Museum.
2) Dr R. W. Rose, Zoology Department, University of Tasmania.

3.2.1b. Macropus eugenii

4 embryos and 23 pouch young aged from 2-18 days were studied (see Appendix). They were supplied, fixed in formaldehyde, by Dr C.H. Tyndale-Biscoe, CSIRO, Division of Wildlife and Rangeland Research, Canberra.

3.2.1c. Potorous tridactylus

Ovaries of 88 pouch young and 12 adults were used (see Appendix). They came from the following sources:

1) Dr Robert Gemmel, Department of Anatomy, University of Queensland.
2) Dr D. Darrell Kitchener, Curator, Western Australian Museum.
3) Dr Megirian, Department of Anatomy, University of Tasmania.
4) Dr R.W. Rose, Zoology Department, University of Tasmania.
5) Dr S.L. Ullmann, Zoology Department, University of Glasgow.
6) The Hill Collection of the Central Embryological Collection of the Hubrecht Laboratory, Utrecht, The Netherlands.

3.2.2. Sexing of pouch young

3.2.2a. Macropus eugenii

The phenotypic sex of the male pouch young was obvious at day 3 of pouch life by the appearance of scrotal anlagen and this is in accordance with Alcorn's (1975) observations. On the other hand, the pouch primordium was visible only at later stages, that is at day 8 of pouch life and thus the younger females were identified by a process of elimination.

3.2.2b. Potorous tridactylus

Determination of chromosomal sex was attempted on a single new born pouch young, using the methods previously described in Section 2.2.3. However these techniques, which worked on the mouse, were unsuccessful and did not yield the sex of the pouch young. Since only a single specimen was available, further experiments to determine the chromosal sex were not possible.

3.2.3. Age determination of pouch young

Where date of birth of pouch young was known, the day of birth was designated as day "0". Age determination was carried out by indirect methods where exact date of birth was unknown. Age was estimated by comparing the linear body measurements and body weights with growth curves prepared by Hughes (1961) and a nanometer designed by Guiler (1960). The linear measurements taken for most pouch young included: crown-rump, manus, head, pes, tail and ear lengths. A table of body weights and measurements has been compiled from the data so obtained (see Appendix C).

3.2.4. Histological procedures

a) The fresh ovaries of pouch young and adult potoroos were dissected out as described in Section 2.2.2. and the gonads were fixed for both light and electron microscopic studies as previously described in Section 2.2.4.

b) The specimens obtained from the Hill Collection had been in alcohol for a long period of time and were therefore soaked in 4% phenol in 70% alcohol for 2-3 days prior to further processing, in order to soften the tissues.

c) The gonads of 2-18 day old wallaby pouch young, which were obtained fixed in formaldehyde, were carefully dissected out with

scissors and fine surgical forceps and washed in several changes of 70% alcohol. They were then dehydrated, cleared and embedded in paraffin wax as described in Section 2.2.4.1a.

d) The caudal parts of the wallaby embryos were dehydrated, cleared and embedded in pure paraffin wax as described in Section 2.2.4.1a with a slightly longer dehydration time, on account of the larger size of the specimens.

e) The gonads were sectioned transversally or longitudinally at 5µm while the caudal parts of pouch young were cut at 8µm. The sections were then stained with Mayer's haemalum and eosin or Mallory's stain, using standard histochemical techniques (Humason, 1979). Mallory's stain is a good general stain especially for collagen and connective tissues. The sections were examined with a Wild M5 microscope.

3.2.5. Photography and Drawings

a) The reproductive tracts of the potoroo pouch young were dissected out and transferred to 70% ethyl alcohol prior to photogarphy. Photographs were taken under incident light only, against a black background, using a Wild M20 photomicroscope and Kodak Panatomic x film. In the case of older pouch young, an Asahi Pentax spotmatic with 105 mm lens and extension bellow was used.

b) The reproductive tracts of the potoroos and wallaby pouch young were drawn using a camera lucida.

The sections were examined using a Wild M20 microscope and photographed using Kodak Panatomic x film.

3.2.6. Estimation of ovarian volumes

Ovarian volumes of 2 adult and 18 pouch young potoroos ranging in age between 1-120 days were estimated, using a morphometric method

developed and applied by Berkwits and Iannaccone (1985) to measure liver patch size in chimeric rats. This method was incorporated into a computer program, written in Basic by Dr D. Neil (Zoology Department, University of Glasgow) and used in the present study. Ovarian volumes were measured as follows:

1) The outlines of individual sections of a serially sectioned ovary were traced by means of a camera lucida attached to a binocular Wild M5 microscope, using magnifications of x60 and x40 for younger and older pouch young respectively. The interval between sampled sections was chosen according to the size of the ovary and the variation in area between consecutive sections. In younger ovaries, cut at 5 μm, every 5th section was measured and in the older ovaries, cut at 8 μm or 10 μm, every 10th, 15th or 20th section was measured. To provide a calibration, a line was traced from a stage micrometer scale at the corresponding magnification.
2) The drawings were positioned on a Summagraphic digitizing tablet connected to a BBC B microcomputer.
3) A cursor was positioned over each end of the calibration line in turn, so that their co-ordinates were entered into the computer program. The real length of the calibration line was also entered from the keyboard. From these values the program calculated a conversion factor to provide the results in real units of measurements.
4) The outlines of the ovaries and, in the case of the older pouch young, the medulla as well were traced by the digitizer cursor key, which streamed co-ordinates into the program.
5) Area computation was performed by the program using an algorithm based upon Green's theorem (Berkwits & Iannaccone, 1985).

The volumes of the truncated cones formed between the adjacent measured sections were calculated. These values were integrated to yield the total volume of the ovary (and medulla when appropriate). Separate values for the volume of the cortex were obtained by subtraction. A growth curve of "regression line" was drawn using a standard programme. The equations are shown in Fig. 3.55, 3.56 and 3.57.

3.3. RESULTS

3.3.1. Early gonad development and sexual differentiation

3.3.1a. Macropus eugenii

3.3.1a.1. Development at day 24 of embryonic life: (Fig. 3.7)

The mesonephroi are large well developed structures. The mesonephric tubules have regular profiles and do not appear to contribute to the gonadal blastema.

The elongated gonad rudiments are invested by a mesothelium which is more or less regular in thickness (1-2 cells thick). It consists of flattened to cuboidal cells, some of which are exhibiting mitosis. The mitotic spindles of the mesothelial cells are perpendicular to the gonadal surface.

The primordial germ cells (PGCs) are larger than the blastema cells, possess vesicular nuclei with beaded chromatin and prominent nucleoli. The blastema is made up of two cell types: a few small fibroblast-like cells and larger irregular cells which form the bulk of the blastema.

3.3.1a.2. Development at day 25 of embryonic life (Fig. 3.8)

No further differentiation is evident over the previous stage except for the arrival of more PGCs in the gonad rudiments.

The mesothelium is several layers in thickness and consists of cuboidal to columnar cells with many PGCs embedded in it. Blood

capillaries with nucleated erythrocytes are found at the hilar region.

3.3.1a.3. 2 day old pouch young

At this stage, the gonad rudiments are compact, shorter and more rounded than at the previous stage. Mitotic figures are found among all cell types. A few mesonephric tubules start to degenerate. Many mitotic figures are observed among the mesothelial cells.

3.3.1a.4. 3 day old pouch young (Fig. 3.9, 3.10 & 3.11)

At day 3 of pouch life, the sex can be distinguished macroscopically in the males by the presence of the scrotal rudiments. The prospective testes are characterized by the appearance of the tunica albuginea and the clumping of the future Sertoli cells to form seminiferous cords. The tunica albuginea is not well developed. It consists of a few small fibroblast-like cells. The testes become more vascularized by the invasion of blood capillaries from the adjacent mesonephroi.

In the presumptive ovaries, on the other hand, no differentiation occurs over the previous stages except for an increase in size due to the mitotic activities of all cell types.

3.3.1a.5. 4-5 day old pouch young

3.3.1a.5.1. The testicular rudiments (Fig. 3.13)

The testicular rudiments become more vascularized with a large blood vessel extending along the length of the gonad within the tunica albuginea. The latter is well developed and consists of several layers of fibroblast-like cells. The mesothelium is regular in thickness with a few cells in mitosis, the spindles being oriented tangentially. A continuous basement membrane lies internal to the mesothelium. The seminiferous cords are also well developed and made up of many dividing germ cells (prespermatogonia) and pre-Sertoli cells, the

latter being randomly distributed within the cords.

3.3.1a.5.2. The ovarian rudiments (Fig. 3.12 & 3.14)

There is no line of demarcation between the mesothelium, which lacks a basement membrane, and the underlying cells.

3.3.1a.6 6-8 day pouch young

3.3.1a.6.1. The testicular rudiments (Fig. 3.15 & 3.17)

The testicular rudiments are well developed and contain seminiferous tubules which, in longitudinal sections, appear to radiate from the hilar region. The seminiferous tubules are surrounded by a basement membrane. Fibroblast-like cells are found in between the seminiferous tubules. A few PGCs are trapped in the mesothelium which is now made up of flattened cells and only a few cuboidal cells. The tunica albuginea consists of several layers of loose fibroblast cells and collagen fibres.

3.3.1a.6.2. The ovarian rudiments (Fig. 3.16)

At day 6 of pouch life, the ovarian rudiment becomes more vascularized but the blood capillaries never reach the mesothelium. Some of the female germ cells still have nuclei with a beaded chromatin arrangement.

3.3.1b. Bettongia gaimardi

3.3.1b.1. 1 day old pouch young (Fig. 3.18)

At birth the sex can be distinguished microscopically. The testicular rudiments are characterized by the development of the tunica albuginea, consisting of a few fibroblast-like cells internal to the mesothelium. At the hilar region small darkly staining cells having irregular nuclei are found. These mesonephric mesenchymal cells can be traced into the gonad and could be migrating from the

mesonephroi.

A few blood capillaries with nucleated erythrocytes have reached the hilar region from the adjacent mesonephroi which are well developed at this stage.

The neonate ovarian rudiments can be recognized by a process of elimination. They are invested by an irregular mesothelium made up of cuboidal to columnar cells. The female germ cells are distinguished by their large size, vesicular nuclei, prominent nucleoli and beaded chromatin distribution. Some of the germ cells are dividing and are found embedded in the mesothelium. The blastema is of 2 cell types: small fibroblast-like cells and large irregular cells possessing irregular nuclei and prominent nucleoli.

3.3.1b.2. 2 day old pouch young (Fig. 3.19)

During the second day of pouch life, the testes are surrounded by a more or less regular mesothelium consisting of cuboidal to flattened cells. A capillary extends along the length of the male gonads within the tunica albuginea. The larger cells of the blastema are clumped together, forming the future seminiferous cords. Within the latter male germ cells, which may be dividing, and a few small fibroblast-like cells are found.

3.3.1c. Potorous tridactylus

3.2.1c.1. 2-3 day old pouch young (Fig. 3.20-3.24)

At birth the testicular rudiment can be distinguished by the development of the tunica albuginea.

During the first few days of pouch life the ovarian rudiment is a compact structure consisting of uniformly distributed germinal and blastemal cells (Fig. 3.20) and invested with an irregular mesothelium which lacks a basal lamina. The mesothelial cells are connected to each other at their free surfaces by junctional complexes such as

zonula occludens or tight junctions, zona adherens or intermediate junctions and macula adherens or desmosomes (Fig. 3.21). The mesothelial cells are cuboidal in shape and a few short microvilli are seen projecting from their free surfaces. The irregularly shaped nuclei may be indented and the chromatin clumps together forming randomly distributed masses. The nucleoli are single. The cytoplasm is rich in scattered ribosomes and small elongated and spherical mitochondria with irregularly arranged cristae. The mitochondria are usually associated with short strands of rough endoplasmic reticulum (ER) which sometimes became U-shaped, surrounding the mitochondria. Small profiles of smooth ER are also found scattered in the cytoplasm. The above described organelles are mostly situated at the apices of the mesothelial cells. At the base of the mesothelial cells, the plasma membrane is highly folded, the processes interdigitating with those from neighbouring cells. Vacuoles of different sizes, some empty others with a dense core, are found near the surface of these mesothelial cells.

The female germ cells (the oogonia), some of which are binucleated, can be distinguished from the surrounding somatic cells on the basis of their size, shape and their specific affinity for the stains used. They are spherical in shape and are larger than the blastema cells (Fig. 3.20 & 3.22). The cell membrane is irregular in outline. Their cytoplasm is more electron dense than that of the stroma, apparently due to the presence of abundant ribosomes and polyribosomes. The cytoplasmic organelles of the oogonia are mostly clustered at one side of the nucleus. These organelles comprise small vesicular or elongated mitochondria with irregularly arranged cristae. In addition, the oogonia are characterized by the presence of a large number of strands of rough ER which may be found in association with

the mitochondria, nuclear envelope or the plasma membrane. Moreover, several profiles of well developed Golgi complex, large lipid droplets and membrane-bound vesicles are also found. The latter vary in size and shape and appear to be either empty or contain an electron dense core. The irregularly shaped nucleus, which may be indented, contains a well developed nucleolus. In the oogonial nucleus the heterochromatin is either restricted to the periphery, forming a beaded configuration, or is distributed throughout the nucleus (Fig. 3.20).

A few germ cells are found in group of 2-3 cells; some may be pyknotic (Fig. 3.23). A few oogonia may possess lysosomes and multivesicular bodies with many vesicles and dense granules.

In the blastema of the ovary, two cell types can be recognized; large cells with more or less spherical nuclei and smaller irregular cells with irregular or elongated nuclei (Fig. 3.20). These cell types differ from each other in the distribution of the heterochromatin in the nucleus. The larger blastema cells are characterized by an even distribution of the heterochromatin, while in the smaller cells it aggregates together forming small masses around the nuclear envelope as well as within the nucleus. Both blastema cell types have small spherical or rod shaped mitochondria, few profiles of Golgi complexes and rough and smooth ER. These organelles, however, are found in larger quantity in the cytoplasm of the small blastema cells. Some of the latter become crescent-shaped, and a few have multivesicular body with a double membrane and several vesicles and cell debris and surround the female germ cells (Fig. 3.24). Invaginations occur in both the cell membrane and the multivesicular bodies. Membrane-bound dense bodies are observed in some blastema cells.

3.3.2. Further differentiation of the ovary

The scheme for a classifiction of follicles in mouse ovaries proposed by Pedersen & Peters (1968) (see Fig. 3.1) is used in this study. According to this the follicles are divided into three main groups: small, medium and large follicles. These authors further subdivide the follicles into eight types according to the number of granulosa cells counted on the largest cross section of the follicle, as follows:

1) small follicles

type 1 - small oocytes with no follicle cells

type 2 - small oocytes surrounded by an incomplete layer of follicle or granulosa cells.

type 3a - oocytes with a complete layer of follicle cells with not more than 20 cells in the largest cross section.

2) medium-sized follicles

type 3b - oocytes surrounded by a complete layer of follicle cells, from 21-60 cells in the largest cross section.

type 4 - oocytes surrounded by a layer of follicle cells, from 61-100 cells in the largest cross section.

type 5a - oocytes surrounded by 3 layers of follicle cells, from 101-200 cells in the largest cross section.

3) large follicles

type 5b - fully grown oocytes surrounded by many layers of follicle cells, from 201-400 cells in the largest cross section, but lacking follicular fluid (i.e. no antrum).

type 6 - large oocytes with many layers of follicle cells from 401-600 cells and several antral cavities.

type 7 - oocytes surrounded by more than 600 cells in the largest cross section with a single fluid filled cavity. These follicles are characterized by the

presence of a cumulus oophorus but without the stalk.

type 8 - is a preovulatory follicle with a single antral cavity and well developed cumulus stalk.

3.3.2a. Macropus eugenii

3.3.2a.1. 9 day old pouch young (Fig. 3.25)

At this stage the blastema has differentiated into two cell types: a few fibroblast-like cells and large cells with irregularly shaped nuclei.

Both the mesothelial cells as well as the female germ cells (oogonia) exhibit mitotic activity. The spindles of the former are either perpendicular to the gonadal surface or tangentially oriented.

In Mallory stained sections, connective tissue fibres and small fibroblasts are evident internal to the mesothelium, forming the tunica albuginea. The mesothelium is made up of 2-3 layers of cuboidal to low columnar cells.

The germ cells are randomly distributed either singly or in pairs.

3.3.2a.2. 11 day old pouch young (Fig. 3.26)

No differentiation over the previous stage was noted except for the presence of dividing oogonia which results in an increase in the size of the ovary.

The large blastemal cells become clumped together forming medullary cords.

3.3.2a.3. 18 day old pouch young (Fig. 3.27)

The ovary can now be distinguished into cortex and medulla separated from each other by an incomplete layer of fibroblast-like cells.

In the cortex, the oogonia are still dividing. Some of them are binucleate and a few are pyknotic. The medullary cords grow and some

of them penetrate the fibrous sheath which delineates the cortex from the medulla.

3.3.2b. Bettongia gaimardi

The timing of events of ovarian development during pouch life is summarized in Table 3.1.

3.3.2b.1. 7 day old pouch young

During the first week of life, the ovary is a compact structure and has the characteristic features of the neonate gonad described above (p. 79).

3.3.2b.2. 14 day old pouch young (Fig. 3.28)

A week later the ovaries increase in size due to the mitotic activities of both blastema and germ cells, the latter giving rise to oogonia. The mesothelium becomes more or less regular in thickness, consisting of a layer of cuboidal to columnar cells with a discontinuous basement membrane. A few pyknotic germ cells are found. The rete is present at the hilar region.

3.3.2b.3. Development between 15-28 days of pouch young (Fig. 3.29 & 3.30)

By day 15 most of the oogonia migrate to the periphery of the ovary forming the cortex. A thick sheath of connective tissue fibres separates the cortex from the medullary region. In the latter, the large cells of the central blastema clump together forming medullary cords in which a few germ cells may be trapped.

3.3.2b.4. 42 day old pouch young (Fig. 3.31 & 3.32)

The ovary is characterized by the appearance of a tunica albuginea made up of fibroblasts, collagen fibres and a few blood capillaries. The mesothelium is regular in thickness and consists of a

layer of cuboidal cells which now rest on a basement membrane. At this stage, meiosis has commenced, the germ cells in the innermost region of the cortex being in various stages of prophase, mostly leptotene and zygotene. Germ cells form groups and then constitute the germ cell nests. A few fibroblasts are found among the cortical germ cell nests.

The medullary cords are found penetrating the fibrous layer which separates the cortex from the medulla. The medullary cord cells are large hypertrophied cells with a nucleus containing several prominent nucleoli. Rete tubules are found at the hilar region.

3.3.2b.5. 56 days old pouch young (Fig. 3.33)

Folliculogenesis is in progress and follicles of types 2 and 3a have developed. Most oocytes are in the prophase of the first meiotic division with some having reached the diplotene stage (resting stage) while a few are pyknotic. The remaining oogonia are found at the periphery of the ovary, below the tunica albuginea. The latter is 2-3 layers in thickness.

The medulla is mainly occupied by medullary cords some of which are located in close vicinity to type 3a follicles.

The mesothelium dips down into and between germ cell nests.

3.3.2b.6. 59 day old pouch young (Fig. 3.34)

The number of follicles formed has increased. Medullary cords are found growing through the connective tissue septae and lying close to the innermost germ cell nests as well as type 3a follicles. A few pyknotic cells are found within the germ cell nests.

3.3.2c. Potorous tridactylus

The timing of events of ovarian development during pouch life is shown in Table 3.2.

3.3.2c.1. Development between 8-15 days of pouch life Fig. 3.35 & 3.36)

At approximately day 8 of pouch life, the PGCs are still migrating to the ovary. They are found at the hilar region and have the same morphological features as those described above for the ovary of the neonate. They are larger in size than the blastema cells, have vesicular nuclei with a beaded heterochromatin arrangement. The ovary cannot, as yet, be regionalised into cortex and medulla. A few blood capillaries are found at the centre of the ovary.

The rete cords were first detected at this stage and are present in the hilar region.

3.3.2c.2. Development at day 32 of pouch life (Fig. 3.37)

The proliferating oogonia become peripherally located at this time, forming the cortex. The irregularly shaped blastema cells clump together in the central or medullary region to form medullary cords. A few groups of oogonia and fibroblasts are also present in the medulla.

The mesothelial cells are cuboidal in shape and more than one layer in thickness, with some mitotic germ cells embedded in them.

3.3.2c.3. Development at day 46 and 48 of pouch life (Fig. 3.38 & 3.39)

At the beginning of this stage, a thin layer of fibroblasts is found separating the newly developed cortex from the medulla. Most of the oogonia, which occupy the cortical region, are found at different stages of mitosis.

During this period of development, the cortex thickens. The majority of oogonia, which form germ cell nests, cease dividing and a few become pyknotic. Fibroblasts and collagen fibres infiltrate between the germ cell clusters. The ovaries at this stage become more vascularized.

The medullary cords are well developed and the rete is present at the hilar region.

3.3.2c.4. Development at day 50 of pouch life (Fig. 3.40)

At day 50 of pouch life the oogonia of the most centrally located germ cell nests, which are closest to the medulla, enter the prophase of the first meiotic division. The divisions are not in synchrony either within a nest or between different germ cell nests. However, the peripherally located oogonia (internal to the mesothelium) are still in mitosis and may be pyknotic.

The tunica albuginea appears in the ovary and consists of a layer of fibroblasts. There is a further infiltration of fibroblasts between the germ cell nests both at the periphery of the cluster and near the medullary region but they do not completely separate the germ cell nests from each other.

The rete is present only at the anterior end of the ovary and is localized to one side.

3.3.2c.5. Development between 51-65 day of pouch life (Fig. 3.41 & 3.42)

The mesothelium becomes more or less regular in thickness and consists of a layer of cuboidal to low columnar cells with oogonia embedded in it.

The majority of the germ cells are now at the prophase of the first meiotic division and a few are still in mitosis. The meiotic divisions are not in synchrony. Atretic oocytes are also found.

The medulla is occupied mainly by well developed medullary cords. In their vicinity rete cords and tubules are found.

3.3.2c.6. Development at day 66-69 of pouch life (Fig. 3.43 a-e)

The only specimen available at this developmental age actually has a chronological age of 86 days (see Discussion, p. 100). The

tunica albuginea is well developed and made up of 1-2 layers of fibroblasts and collagen fibres.

The deeper oocytes, those near the medulla, reach the diplotene stage of meiosis (type 2 follicles) but are scarce. In 1um thick sections stained with toluidine blue, the oocyte cytoplasm is characterized by the presence of darkly stained granules (probably lipid droplets). Similar granules could be seen in the cytoplasm of the interstitial cells.

At the ultrastructural level, the oocyte cytoplasm appears to be less electron dense than the nucleus and the surrounding follicle cells. However, the cytoplasm looks dark in the region where most of the organelles are grouped together, at one side, forming a crescent shaped structure. The aggregate comprises several profiles of Golgi complexes, rough and smooth ER, polyribosomes and mitochondria. The latter have tubular cristae with an enlarged lumen separating the two leaves of the cristae. In some of these mitochondria, the cristae are found at the polar ends of the organelles.

The oocytes are surrounded by an incomplete layer of granulosa cells. The latter are irregular in shape, have elongated nuclei and a prominent nucleolus. They also possess elongated and spherical mitochondria with many irregularly arranged cristae, several profiles of Golgi complexes, a few lysosomes and rough ER which may surround the mitochondria.

The medullary cords clump together and lose their basal lamina at the point of contact. At this stage, electron micrographs indicate the presence of little smooth ER or lipid droplets in the cytoplasm of the medullary cord cells. The lipid droplets are associated with the mitochondria which are spherical or rod shaped having irregularly arranged cristae.

3.3.2c.7. Development between 70-75 days of pouch life (Fig. 3.44 & 3.45)

The medullary cords grow and penetrate the fibrous layer between the cortex and medulla and enclose oocytes forming type 3 follicles. The rete is branched and grows deeper into the ovary and makes contact with the medullary cords.

Folliculogenesis is in progress and follicles of type 3a are found. The cells of medullary cords in the vicinity of the oocytes dissociate and give rise to granulosa cells. POFs of type 3a with 2-4 ova are found. They may be in direct contact with each other or separated by granulosa cells.

3.3.2c.8. Development at day 82 of pouch life (Fig. 3.46 a-f)

The thick cortex is mainly occupied by type 2 follicles and the only germ cell nests still remaining are in the hilar region. Those oocytes which are trapped in the medullary cords and rete are found to be degenerating. Folliculogenesis continues and follicles type 3a, 3b and 4 and 5 are all present. A POF of type 4 with 20 oocytes was found in one ovary.

3.3.2c.9. Development between 91-92 days of pouch life (Fig. 3.47 a-b, 3.48 a-c & 3.49 a-m)

The ovary is characterized by a cortical zonation: the peripheral zone, internal to the mesothelium, consists of oocytes at different stages of meiosis; central to this zone lie type 2 follicles in 1-2 layers. Follicles type 3a, 3b and 4 are all present at the corticomedullary region and in the medulla. Some follicles of type 3a and 3b are polyovular and consist of 2-4 oocytes.

At this stage more type 5a & b and a few type 4 follicles are developed. They are surrounded by a capsule of modified stromal cells called theca folliculi, which is differentiated into an inner vascular

layer, the theca interna, and an outer layer, the theca externa. The theca interna consists of irregularly shaped hypertrophied cells and coursing in between are blood vessels and capillaries. The theca externa, on the other hand, is composed of a few fusiform cells scattered between the closely packed collagen fibres. Some of the type 3b, 5a and 5b follicles are polyovular and the latter were observed to contain up to 10 oocytes. The oocytes within the polyovular follicles may be of different sizes. They are either in close contact with each other or they may be separated by granulosa cells.

In the cortico-medullary junction as well as in the medulla follicles with fluid-filled cavities or antra are found. These large follicles are known as vesicular or antral follicles (type 6 follicles). The interstitial tissue is first recognizable at this stage. The interstitial tissue cells are hypertrophied and found in groups separated from each other by a thin layer of collagen fibres.

3.3.2c.10. Development at day 98 of pouch life (Fig. 3.50 a-e)

Atresia is prominent at this stage. Some atretic type 2 follicles are found among the mesothelial cells, while a few are scattered elsewhere in the cortex and in the hilar region. Types 3b and 4 follicles, in different stages of atresia, are observed in the cortico-medullary region as well as in the medulla.

The rete ovarii is present and ramifies in the anterior region of the medulla.

3.3.2c.11. Development between 100-120 days of pouch life (Fig. 3.51 a-d)

The mesothelium is regular and consists of a layer of cuboidal to flattened cells. Follicles of type 2 are frequent and predominate the cortex at this stage. A few binucleate, trinucleate or quadrinucleate oocytes may be found in these follicles. Types 3a, 3b, 4, 5a and 5b follicles are also present. By day 120 of pouch life some of the

latter are degenerating. POFs of types 4, 5a and 6 containing 3-4 oocytes also occur. The rete is well developed and found in the hilar region, but only remnants of the medullary cords still exist.

3.3.2c.12. Ovary structure of adult potoroos (Fig. 3.52, 3.53 & 3.54)

Ovaries from 12 females ranging in age between less than 2 years to 9 years were examined. One of these females which was 2.5 years old was lactating.

In the adult ovaries the mesothelium is regular, consisting of a layer of cuboidal cells with a thin basement membrane. The tunica albuginea is made up of 3-4 layers of fibroblasts and collagen fibres.

Both folliculogenesis and atresia are in progress: a few isolated groups of type 2 follicles are found in the cortex central to the tunica albuginea and some are trapped in the latter and in the mesothelium. Follicles of types 3a, 4 and 5a are observed at the cortico-medullary junction. In the medulla several type 5a, 5b and a few type 6 follicles are present. Type 7 and 8 follicles may also be detected in some ovaries, the latter being situated in the cortical region, just internal to the tunica albuginea. In the oldest female, the cortex is mainly occupied by stromal cells with no follicles present. Follicles at various stages of development are also found degenerating in the adult ovaries.

Two kinds of interstitial tissue (IT) can be distinguished in adult ovaries: these are the medullary cord and thecal type IT (see p. 22). In the younger females a few clusters of medullary cord IT, are present. The medullary cord IT cells are hypertrophied, glandular in appearance and found in groups separated from each other by a thin layer of collagen fibres and a few blood vessels. Their nuclei are spherical in shape with one or more prominent nucleoli.

Subsequently the IT of medullary cord origin disappears from the ovaries of older animals. The ovarian medulla becomes occupied by another type of IT, probably derived from the atretic follicles (thecal type) as atresia of medium-sized and large follicles is prominent. The thecal IT differs from the medullary derived IT in appearance: the cytoplasm of the cells is more vacuolar and the cluster organisation of medullary cord-derived IT disappears. The IT of the lactating female was less conspicuous.

The older ovaries have obviously undergone ovulation since they contain a number of corpora lutea. These are composed of large polyhedral cells with vesicular nuclei (the granulosa lutein cells) and smaller cells with dense nuclei (the theca lutein cells) and blood capillaries.

In the centre of the ovary of the lactating female a corpus albicans (a degenerating corpus luteum) was observed. It was smaller than the corpora lutea and contained more blood capillaries. In older ovaries corpora albicantia were absent and this is probably due to their degeneration.

The adult ovaries are highly vascularised: large veins and arteries being found in both the cortex and the medulla.

Table 3.1 Timetable of gonad development in bettongs

Age (days)	Events in gonad development
Neonate	Indifferent gonad, PGCs dividing asynchronously.
2 days	Sex differentiation initiates in males. Tunica albuginea and prospective seminiferous cords develop.
7 days	Female gonads start to differentiate. Nucleated erythrocytes. No trace of tunica albuginea, blastema differentiates into small fibroblast-like cells and larger cells (medullary cord cells).
14 days	Ovary increases in size as a result of the mitotic activity of the PGCs. Mesothelium is more or less regular in thickness. A few pyknotic germ cells are found. Some nucleated erythrocytes are still present. Rete observed at hilar region.
15 days	Oogonia (female germ cells) become peripherally located forming the cortex. Majority of germ cells are dividing. Fibrous layer separates the cortex from the medulla. Medullary cords are formed. Erythrocytes are enucleated.
42 days	Tunica albuginea present in the ovaries. Oocytes at various stages of meiosis but a few oogonia still present. Medullary cords penetrate fibrous layer which separates the cortex from the medulla. Rete tubules in hilar region.
56 days	Folliculogenesis in progress; Follicles of type 2 and 3a are developed.
59 days	More follicles (type 2) are formed. A few oocytes become pyknotic.

Table 3.2 Timetable of ovary development in potoroos

Age (days)	Events in ovary development
2-3 days	The female gonad rudiments differentiate, the blastema giving rise to two cell types: small fibroblasts and larger irregular cells with more or less spherical nuclei. Erythrocytes still nucleated. The female germ cells are spherical in shape and are larger than the blastema cells.
8-15 days	Oogonia dividing. Rete first detected at this stage.
32 days	The ovary can be distinguished into cortex and medulla. The large irregular cells clump together forming the medullary cords.
48 days	A thin layer of connective tissue appears between the cortex and medulla. Oogonia which form germ cell nests cease dividing and a few become pyknotic. The ovary becomes more vascularized.
50 days	Oogonia enter the prophase of the first meiotic division to form oocytes. Tunica albuginea is present.
51-65 days	The majority of the germ cells have reached the first meiotic division.
66-69 days	Folliculogenesis begins: follicles of type 2 are developed. The medullary cords grow and penetrate the fibrous layer. The rete is branched and makes contact with the medullary cords.
70-75 days	Folliculogenesis is in progress; follicles of type 3a & 3b, some of which are polyovular, are developed.
82 days	Type 3b follicles (bi- or triovular), type 4 and 5 are all developed.
91-92 days	The ovary is characterized by the presence of cortical zonation, the peripheral zone consisting of meiotic oocytes while follicles of type 2 constitute the inner region. Type 6 follicles developed. POFs (type 3a, 3b) are frequent and may contain 2-5 oocytes. Medullary cords give rise to interstitial tissue.
98 days	Atresia of the various follicle stages is prominent.
100-120 days	Follicles of type 2 are frequent and predominate in the cortex. Types 4, 5a and 6 follicles, some polyovular having 4-5 oocytes, are found. Remnants of medullary cords are detected. Interstitial tissue present.
Mature ovaries	Tunica albuginea is 3-4 cell layers in thickness. Follicles are few. Remnants of medullary cords and a few clumps of interstitial tissue are found in the younger ovaries but disappear from the older ovaries. Corpora lutea may be present.

Abbreviations

A	small fibroblast like cells
af	atretic follicle
an	antrum
ao	atretic oocyte
B	large blastema cell
b	blastema
bl	basal lamina
bo	binucleate oocyte
c	cortex
cd	cell debris
cl	corpus luteum
d	desmosome
e	erythrocyte
er	rough endoplasmic reticulum
f	follicle
fl	fibrous layer
G	Golgi complex
g	granulosa cell
gc	germ cell
gcn	germ cell nest
gl	granulosa layer
gr	gonadal ridge
h	hilar region
hg	hindgut
ic	incipient cortex
it	interstitial tissue
l	lipid droplet
lv	liver
m	mesothelium
mb	membrane bound dense body
mc	medullary cord
mcc	medullary cord cell
md	medulla
mf	mitotic figure
mgc	mitotic germ cell
mi	mitochondria
ms	mesonephros
msg	mesonephric glomerulus
mst	mesonephric tubule
mt	metanephric rudiment
mv	microvilli
mvb	multivesicular body
n	nucleus
nt	notochord
nu	nucleolus
o	oogonium
oc	oocyte
od	oocyte at diplotene
ol	oocyte at leptotene
op	oocyte at pachytene
or	ovarian rudiment
ov	ovary
oz	oocyte at zygotene
p	pancrease
pof	polyovular follicle
pr	polyribosome
pgc	primordial germ cell
pS	pre-Sertoli cell
r	rete
rt	rete tubule
s	stroma
sc	sex cord
st	seminiferous tubule
t	testis
ta	tunica albuginea
th	theca layer
tr	testicular rudiment
2f	type 2 follicle
3af	type 3a follicle
3bf	type 3b follicle
4f	type 4 follicle
5af	type 5a follicle
5bf	type 5b follicle
6f	type 6 follicle
7f	type 7 follicle
Zo	Zonula occludens

For all abbreviations see page 97

Fig. 3.1. A scheme for classification of follicles (from Pedersen & Peters, 1968).

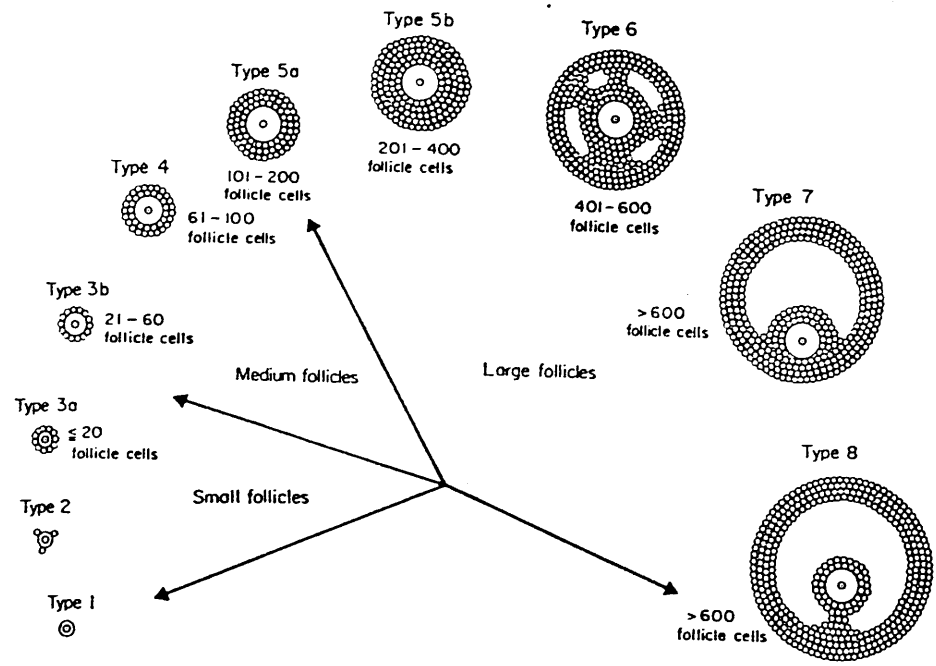

Type 5b
Type 6
Type 5a
201 – 400
follicle cells
Type 4
101 – 200
follicle cells
401 – 600
follicle cells
Type 7
61 – 100
follicle cells
>600
follicle cells
Type 3b
21 – 60
follicle cells
Medium follicles
Large follicles
Type 3a
≤ 20
follicle cells
Type 8
Small follicles
Type 2
Type 1
>600
follicle cells

Fig. 3-1

Fig. 3.2. Adult female potoroo.

Fig. 3.3. 2-3 day old pouch young potoroo. (Bar = 7 mm).

Fig. 3.2

Fig. 3.3

Fig. 3.4. Adult tammar wallaby. (From Lyne, 1967).

Fig. 3.5. Pouch young tammar wallaby. (Bar = 7 mm).

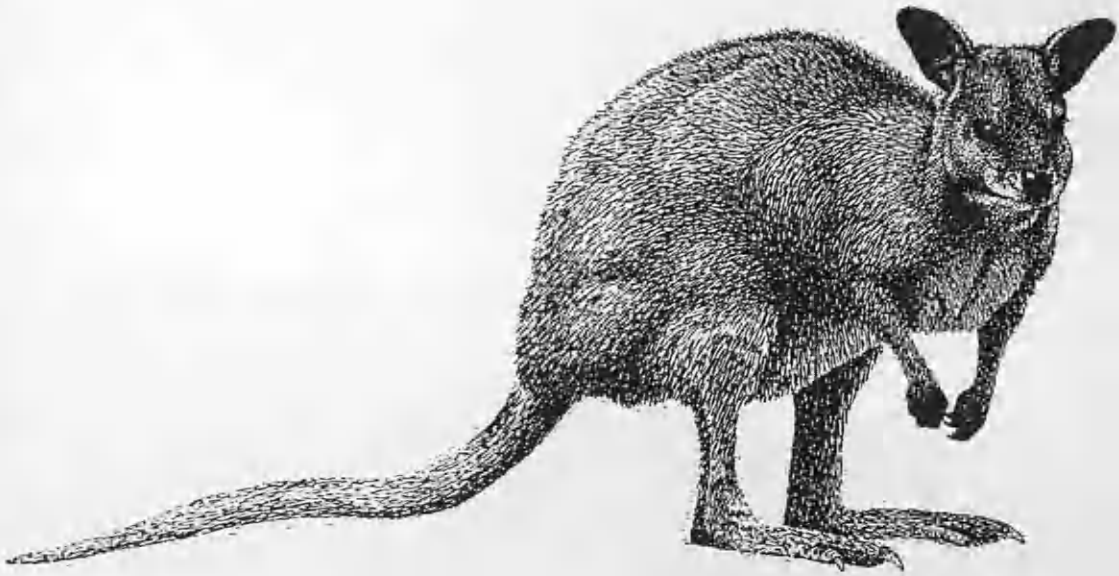


Fig. 3·4

Fig. 3·5

Fig. 3.6. Pouch young bettong. (Bar = 4 mm).

Fig. 3.6

Fig. 3.7a. Transverse section through the posterior region of 24 day old tammar wallaby embryo. Note gonadal ridge (gr) mesonephros (ms), metanephric rudiment (mt), hindgut (hg), liver (lv), (Bar = 100 μm).

Fig. 3.7b. Detail of Fig. 3.7a at the region of the gonadal ridge, showing primordial germ cells (pgc) with beaded chromatin. (Bar = 50 μm).

Fig. 3.8. Transverse section through the gonadal rudiment of a 25 day old tammar embryo. Note multilayered mesothelium (m) and primordial germ cells (pgc). (Bar = 50 μm).

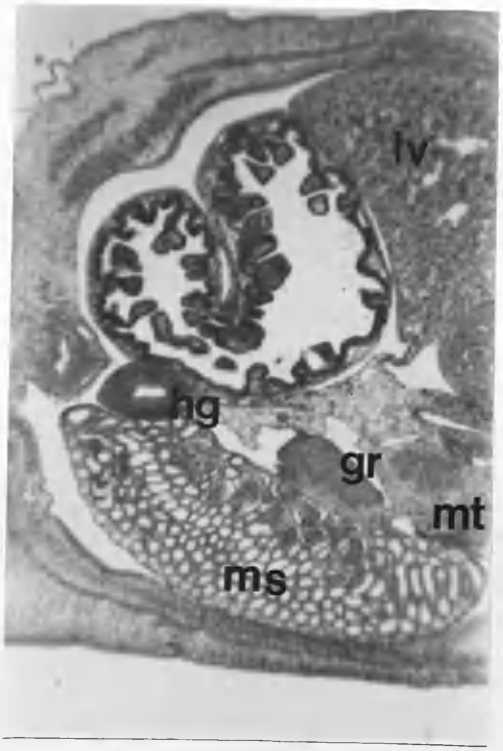


Fig. 3·7a

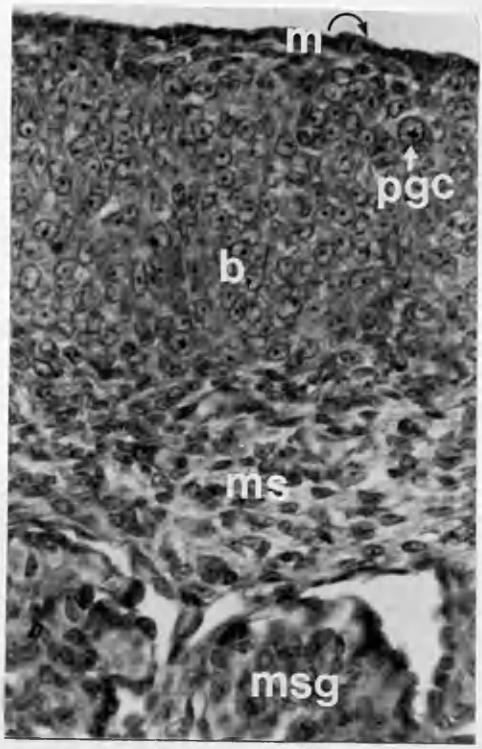


Fig. 3·7b

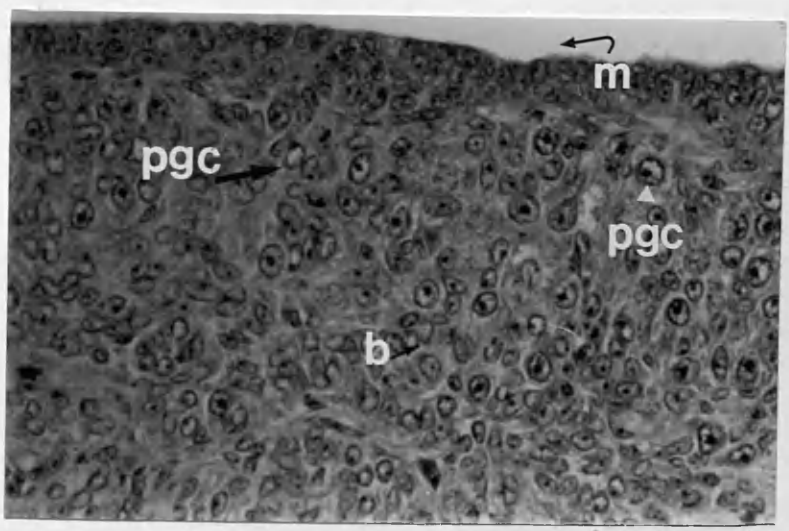


Fig. 3.8

Fig. 3.9. Transverse section through the testicular rudiment (tr) and the mesonephros (ms) of a 3 day old pouch young tammar. (Bar = 500 μm).

Fig. 3.10. Transverse section through the testicular rudiment of a 3 day old pouch young tammar. Note the presence of the tunica albuginea (ta), the clumping of primordial germ cells (pgc) and the pre-Sertoli cells (pS) to form sex cords (sc). (Bar = 100 μm).

Fig. 3.11a. Longitudinal section through the ovarian rudiment of a 3 day old pouch young tammar. (Bar = 100 μm).

Fig. 3.11b. Detail of Fig. 3.11a. Note compact structure and mitotic figure (mf) in the mesothelium (m) and mitotic germ cells (mgc). (Bar= 50 μm).

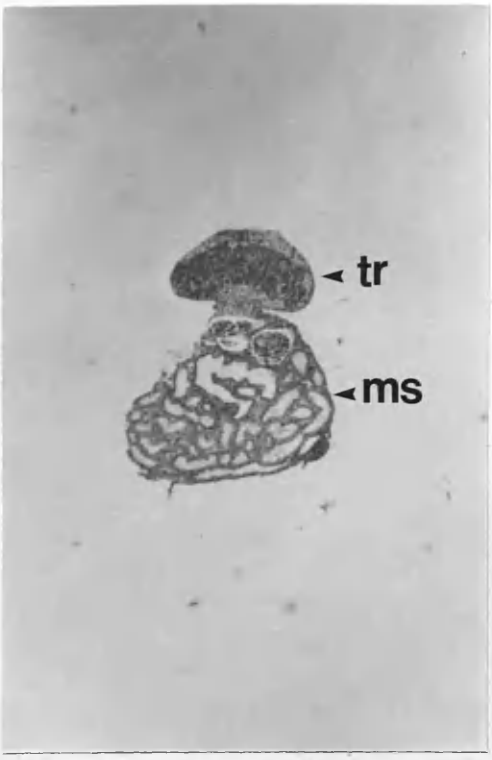


Fig. 3.9

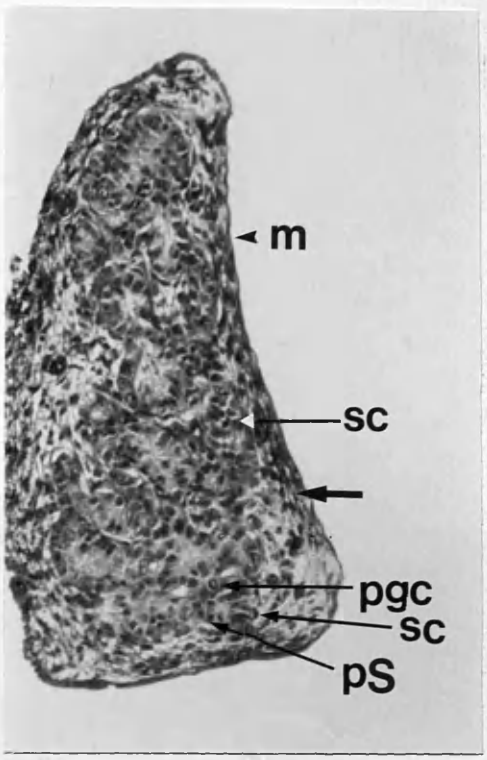


Fig. 3·10

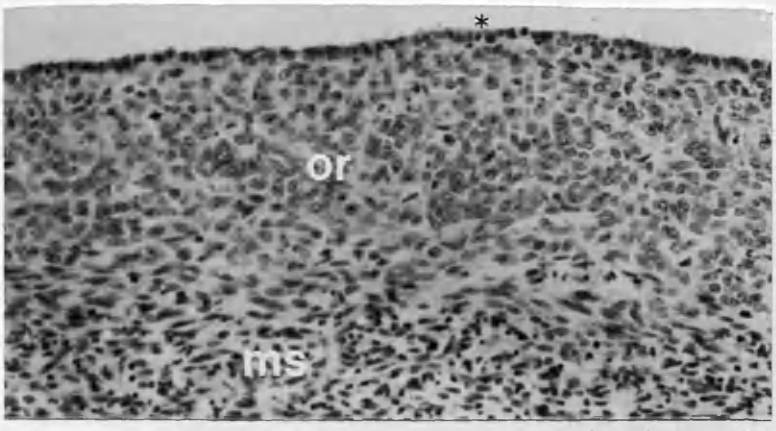


Fig. 3·11a

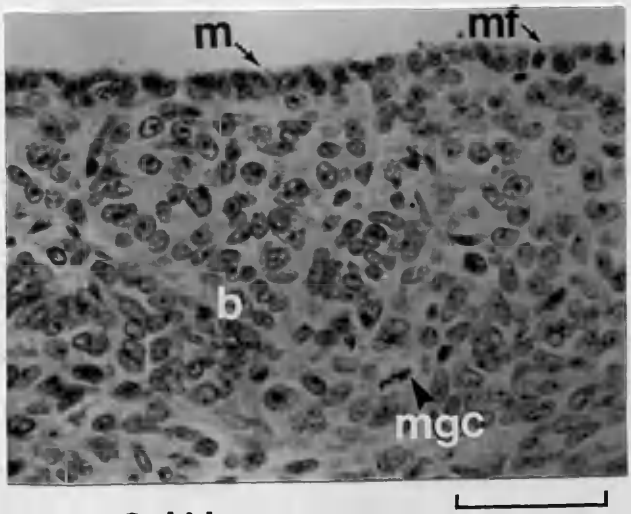


Fig. 3·11b

Fig. 3.12a. Longitudinal section through the ovarian rudiment of a 4 day old tammar. (Bar = 100 μm).

Fig. 3.12b. Detail of Fig. 13.12a. Note lack of cellular differentiation. (Bar = 50 μm).

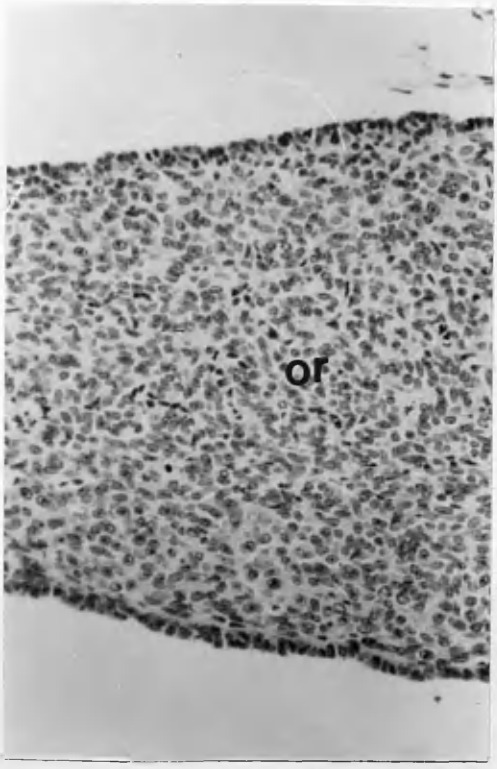


Fig. 3·12a

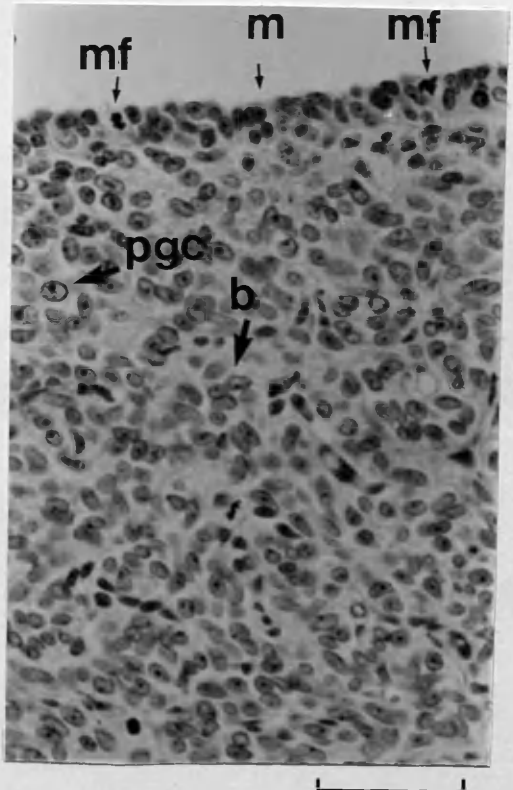


Fig. 3·12b

Fig. 3.13a. Transverse section through the testicular rudiment of a 5 day old tammar. (Bar = 100 μm).

Fig. 3.13b. Detail of Fig. 3.13a. Note flattened mesothelial cells (m) and sex cords (sc). (Bar = 50 μm).

Fig. 3.14b. Detail of Fig. 3.14a. (Bar = 50 μm).

Fig. 3.14a. Transverse section through the ovarian rudiment of a 5 day old tammar. (Bar = 100 μm).

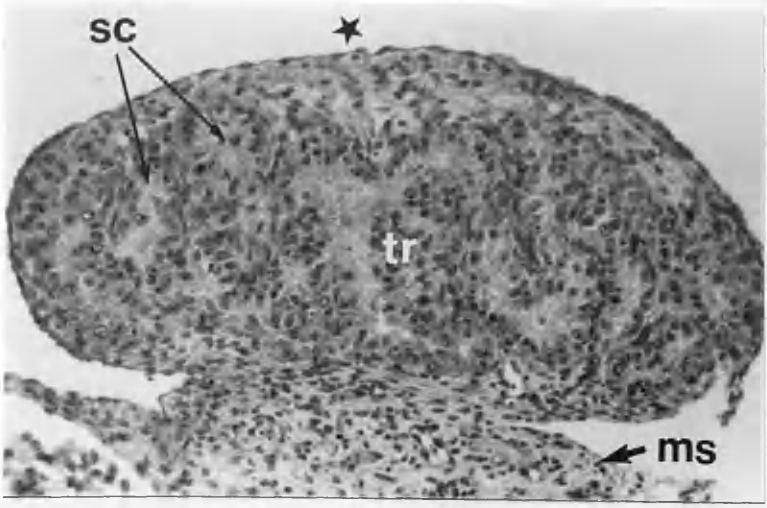


Fig. 3·13 a

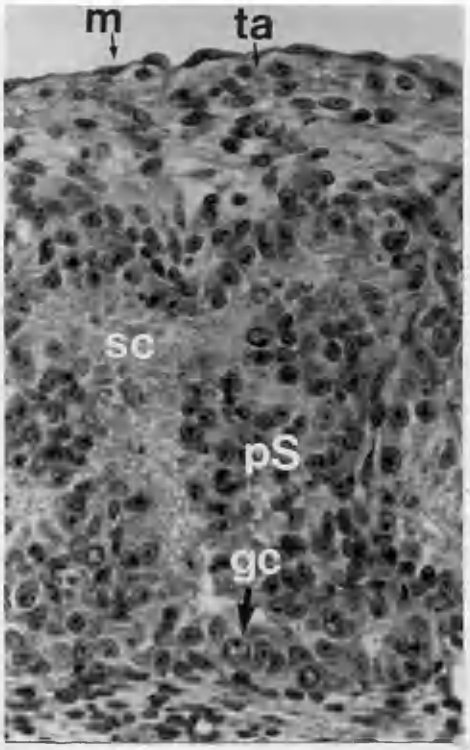


Fig. 3·13b

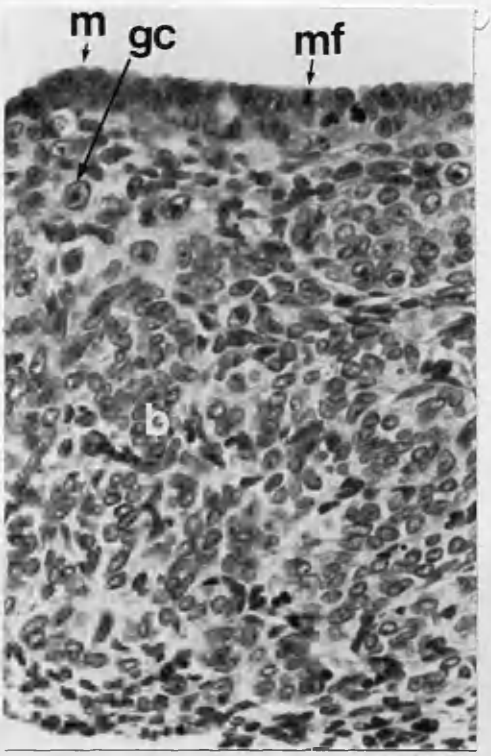


Fig. 3·14b

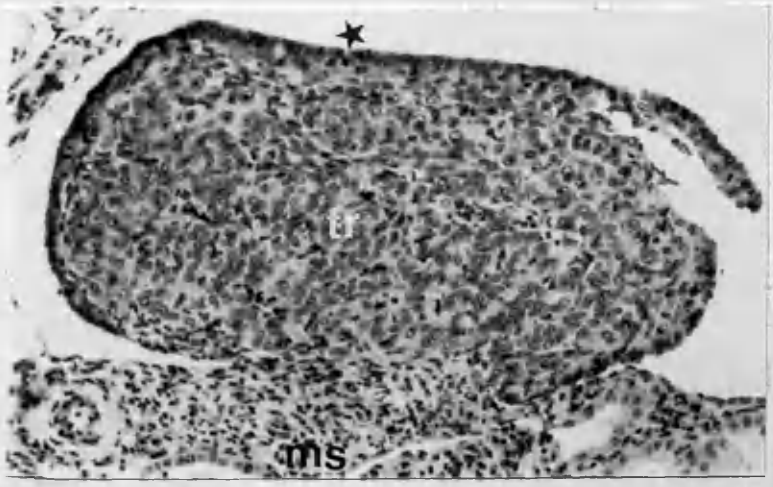


Fig. 3·14 a

Fig. 3.15a. Transverse section through the testicular rudiment of a 6 day old pouch young tammar. Note the seminiferous tubules (st). (Bar = 100 μm).

Fig. 3.15b. Detail of Fig. 3.15a showing the mesothelium (m) tunica albuginea (ta) and the seminiferous tubules (st). (Bar = 50 μm).

Fig. 3.16b. Detail of Fig. 3.16a. Note the lack of cellular differentiation and the mesonephric glomerulus (msg). (Bar = 100 μm).

Fig. 3.16a. Transverse section through the ovarian rudiment and the mesonephros of a 6 day old pouch young tammar. (Bar = 500 μm).

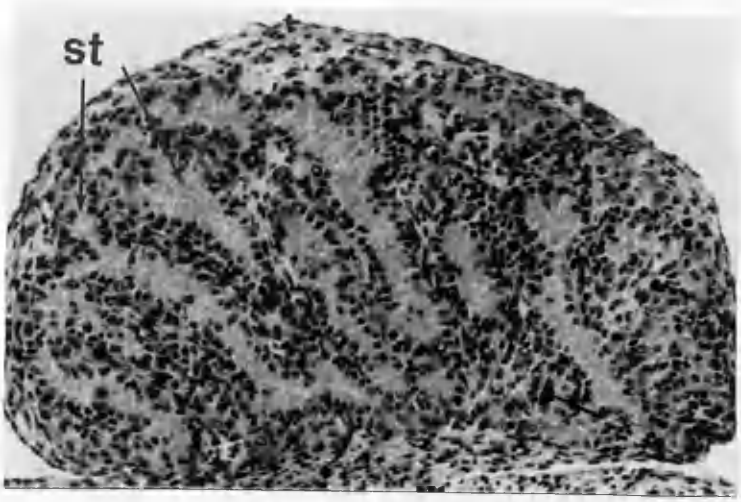


Fig. 3.15a

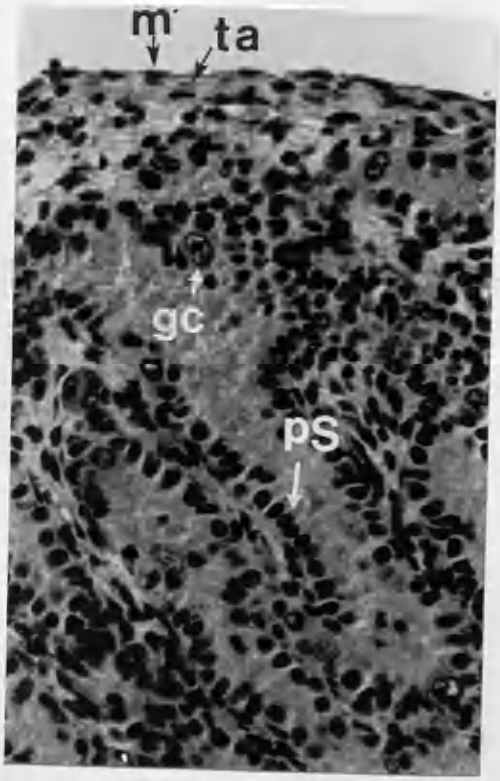


Fig. 3.15b

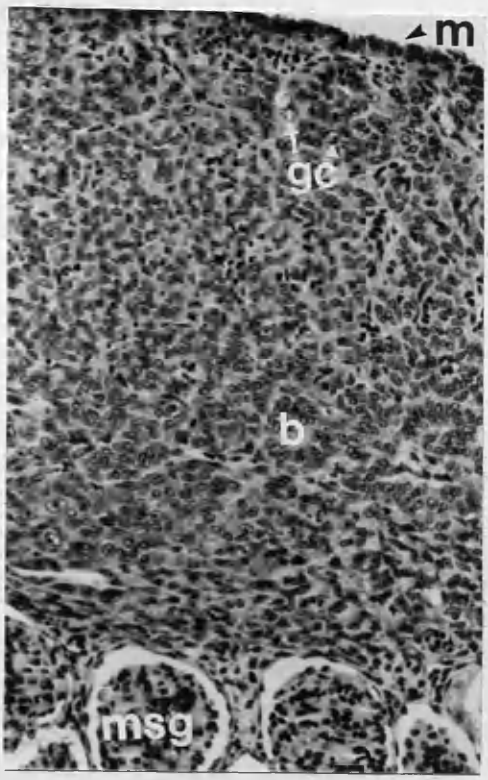


Fig. 3.16b

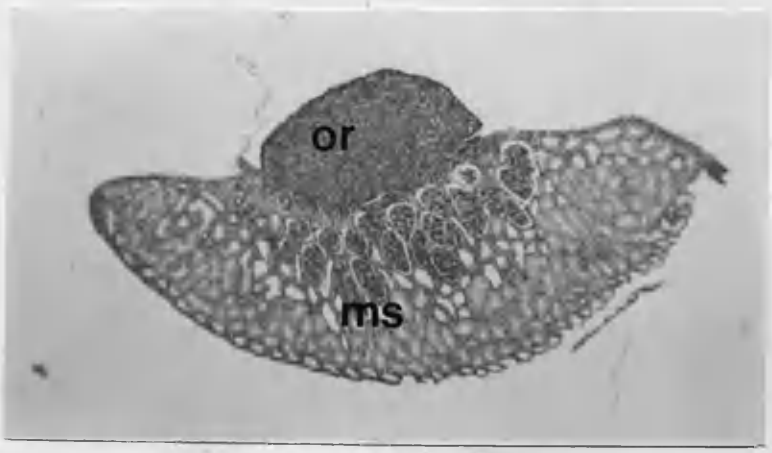


Fig. 3.16a

Fig. 3.17a. Transverse section through the testicular rudiment of an 8 day old tammar. (Bar = 100 μm).

Fig. 3.17b. Detail of Fig. 3.17a. (Bar = 50 μm).

Fig. 3.17a

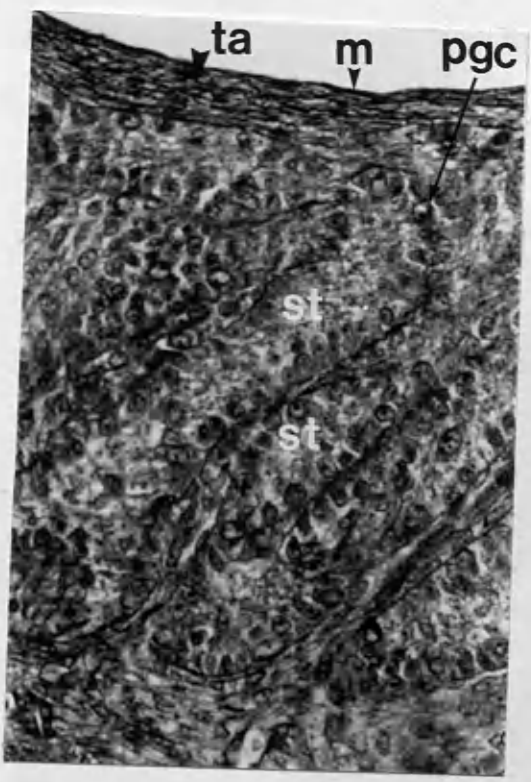


Fig. 3.17b

Fig. 3.18a. Transverse section through the testicular rudiment and the mesonephros of a neonate bettong. (Bar = 100 μm).

Fig. 3.18b. Detail of Fig. 3.18a. Note the mesothelium (m), tunica albuginea (ta) and the primordial germ cells (pgc). (Bar = 25 μm).

Fig. 3.19. Transverse section through the testicular rudiment of a 2 day old pouch young bettong. Note the development of the sex cords (sc). (Bar = 100 μm).

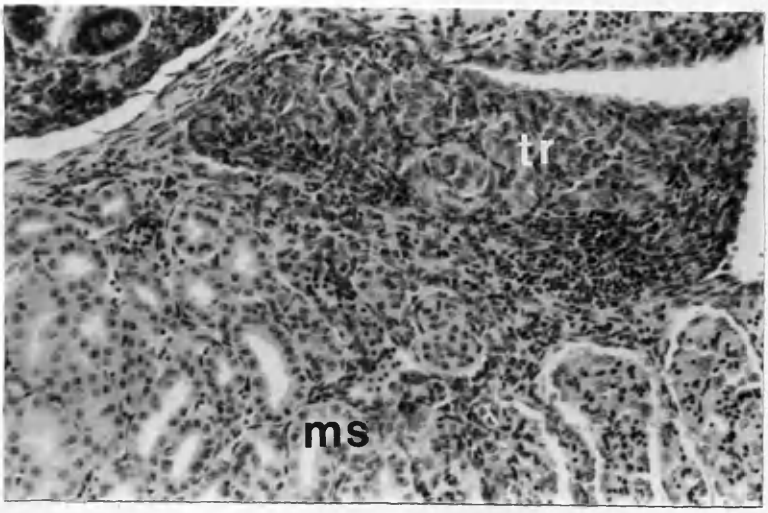


Fig. 3.18 a

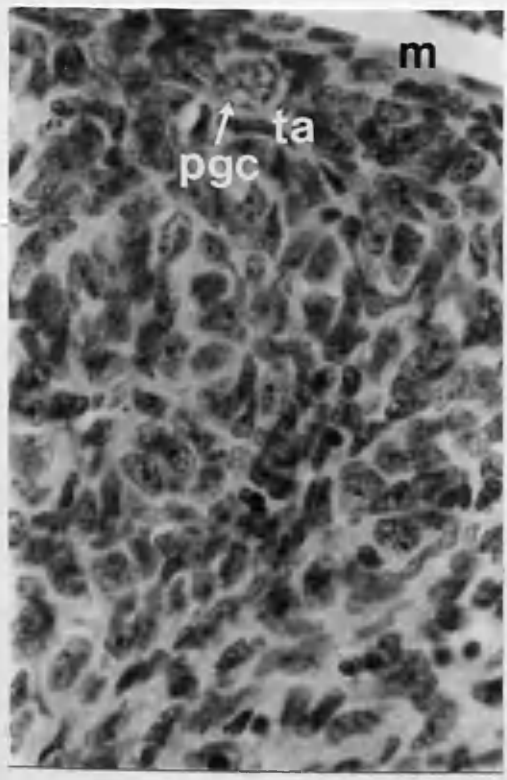


Fig. 3.18b

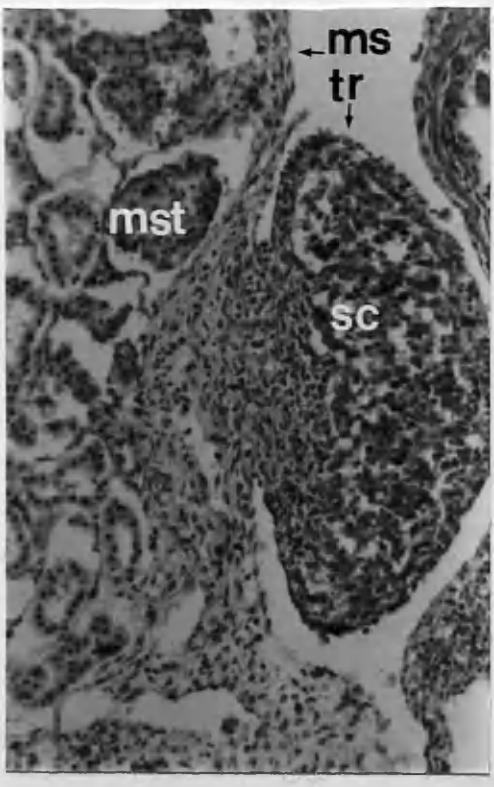


Fig. 3.19

Fig. 3.20. Electron micrograph through the ovarian rudiment of a 2-3 day old pouch young potoroo, showing the female germ cells (gc) and the blastema (b) differentiating into two cell types (A+B). x 2.4 K.

Fig. 3.21. Same gonad as in Fig. 3.20, showing the mesothelial cells (m). x 9.45 K.

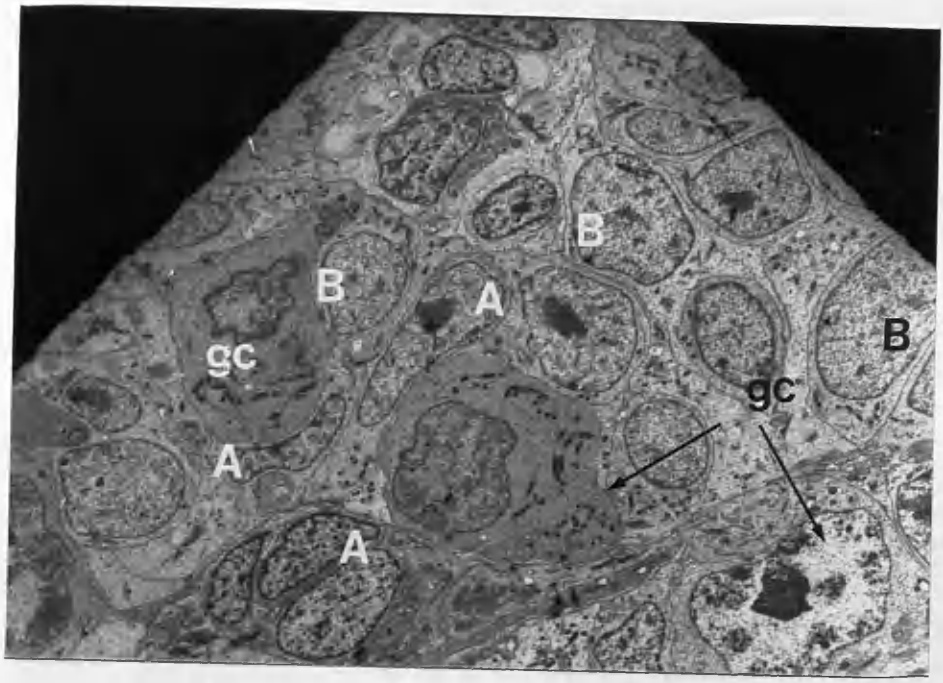


Fig. 3.20

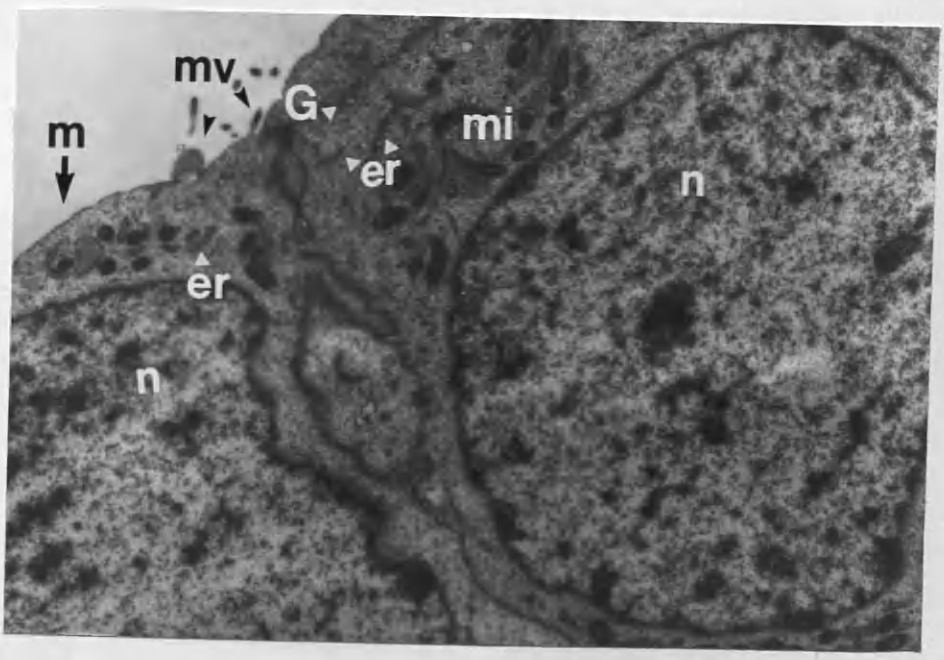


Fig. 3.21

Fig. 3.22. Same gonad as in Fig. 3.20, to show a germ cell (gc) and a blastema cell (b). x 5.6 K.

Fig. 3.23. Same gonad as in Fig. 3.20. Note germ cells (gc) at early stages of atresia. x 5.6 K.

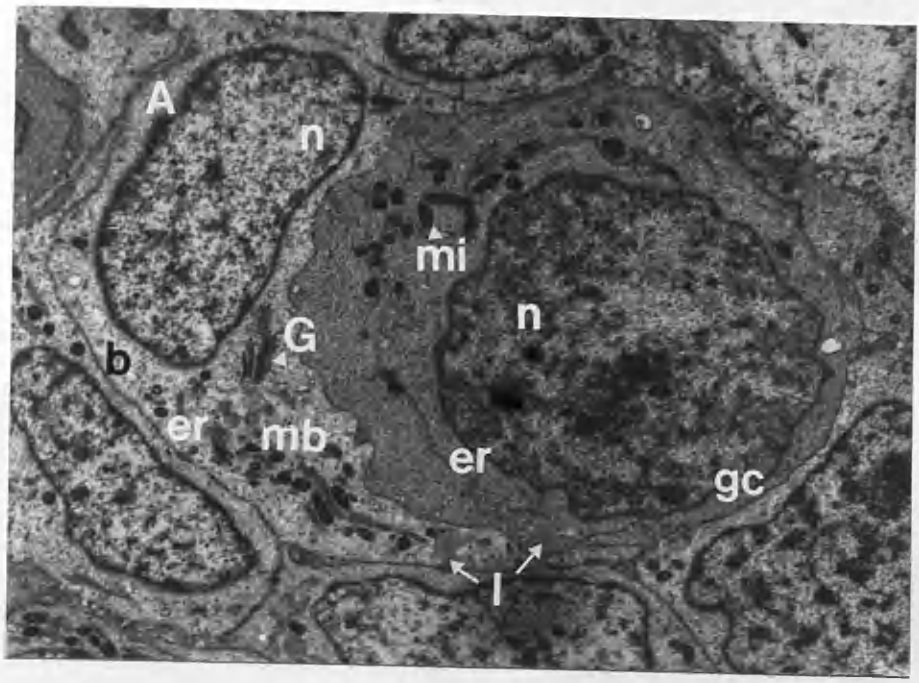


Fig. 3·22

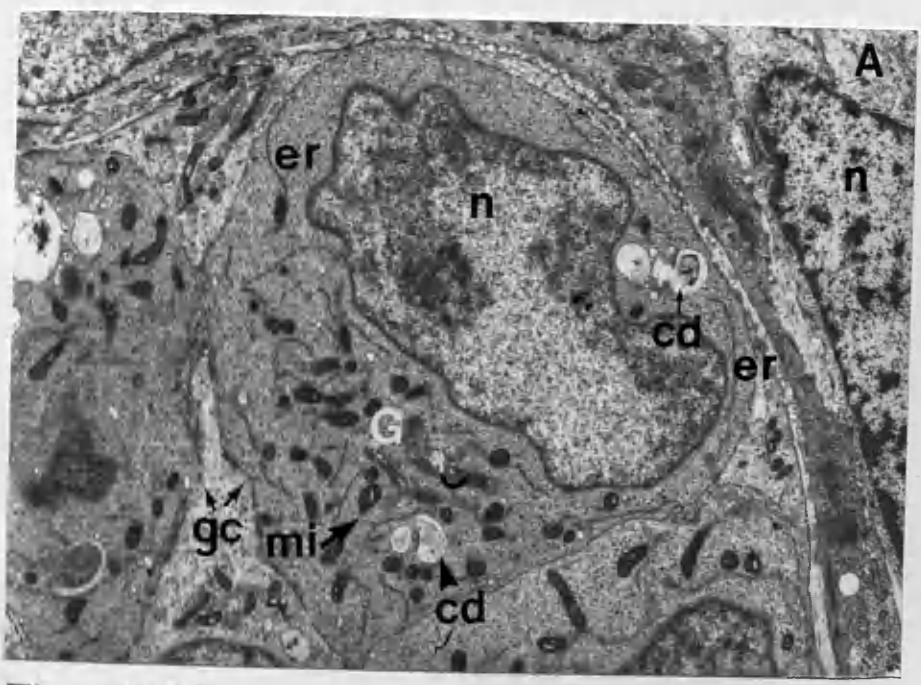


Fig. 3·23

Fig. 3.24. Same gonad as in Fig. 3.20, showing a blastema cell (b) with a multivesicular body (mvb). x 24 K.

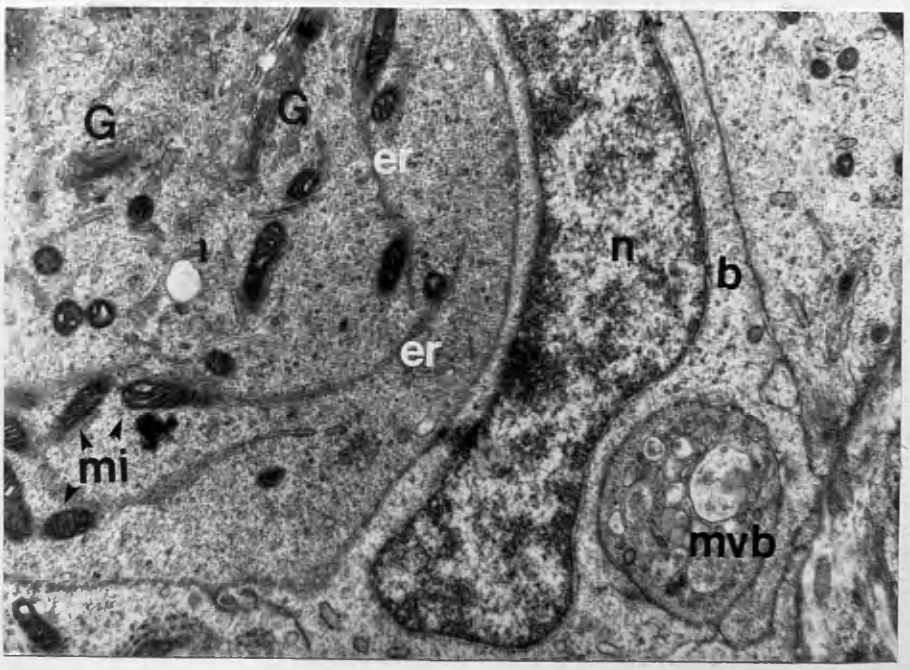


Fig. 3.24

Fig. 3.25a. Transverse section through the ovary of a 9 day old pouch young tammar. (Bar = 100 μm).

Fig. 3.25b. Detail of Fig. 3.25a. Note mitotic figures in the mesothelium (m) and the blastema differentiating into small fibroblast-like cells (A) and large cells (B). (Bar = 50 μm).

Fig. 3.26a. Transverse section through the ovary of an 11 day old pouch young tammar. Note the regular mesothelium (m) and clumping of large cells to form medullary cords (mc). (Bar = 100 μm).

Fig. 3.26b. Detail of Fig. 3.26a to show the medullary cord cells (mcc) with irregular nuclei. (Bar = 50 μm).

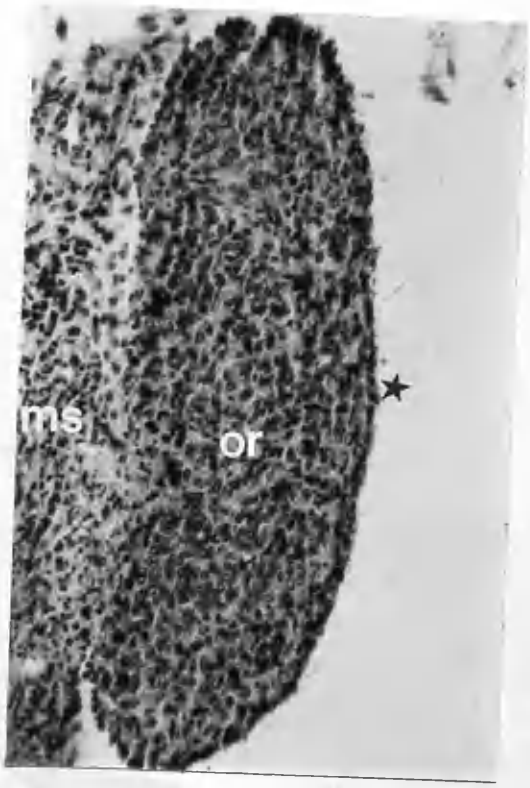


Fig. 3.25a

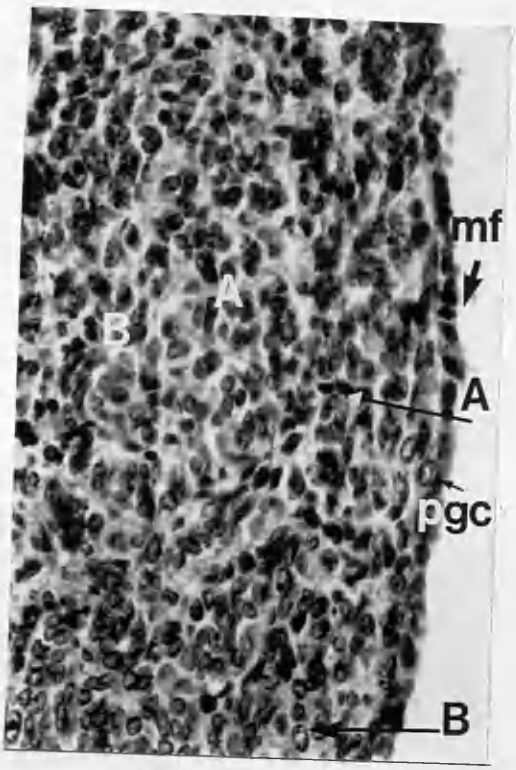


Fig. 3.25b

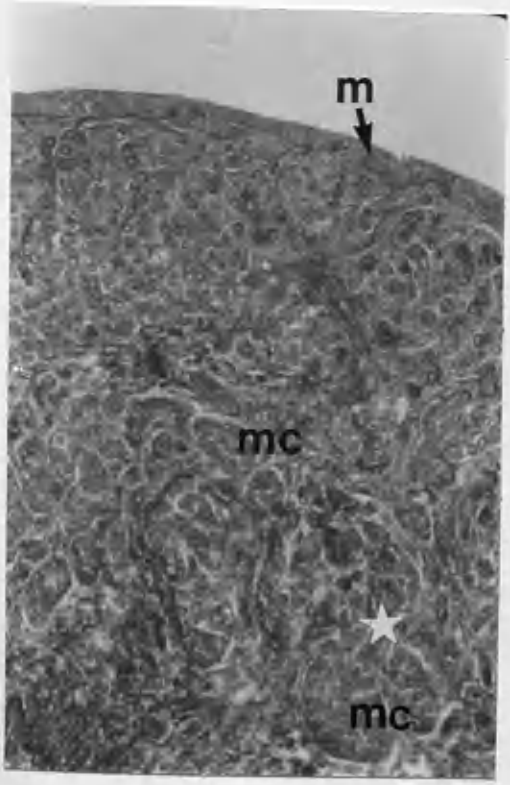


Fig. 3.26a

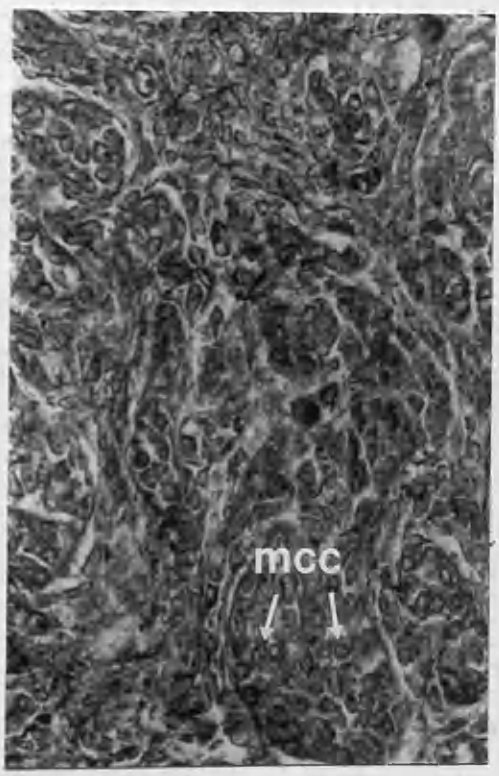


Fig. 3.26b

Fig. 3.27a. Transverse section through the ovary of an 18 day old pouch young tammar. (Bar = 100 μm).

Fig. 3.27b. Detail of Fig. 3.27a. Note mitotic germ cells (mgc) and the appearance of a fibrous layer (fl) between the incipient cortex (ic) and medulla (md). (Bar= 50 μm).

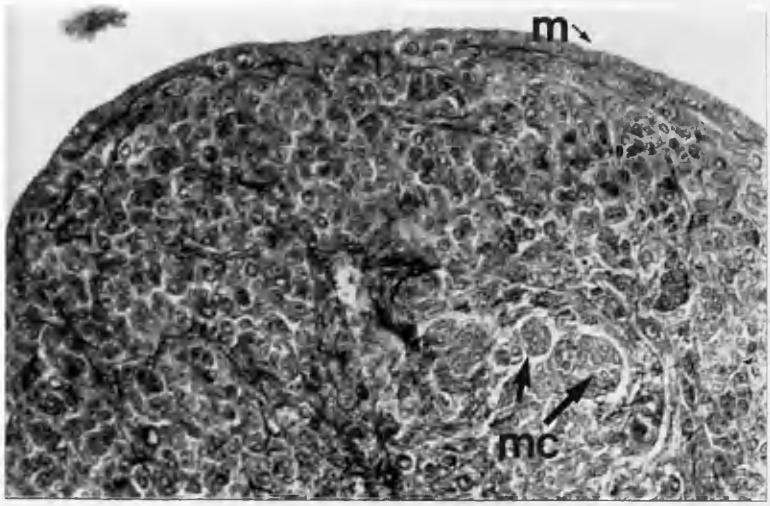


Fig. 3.27a

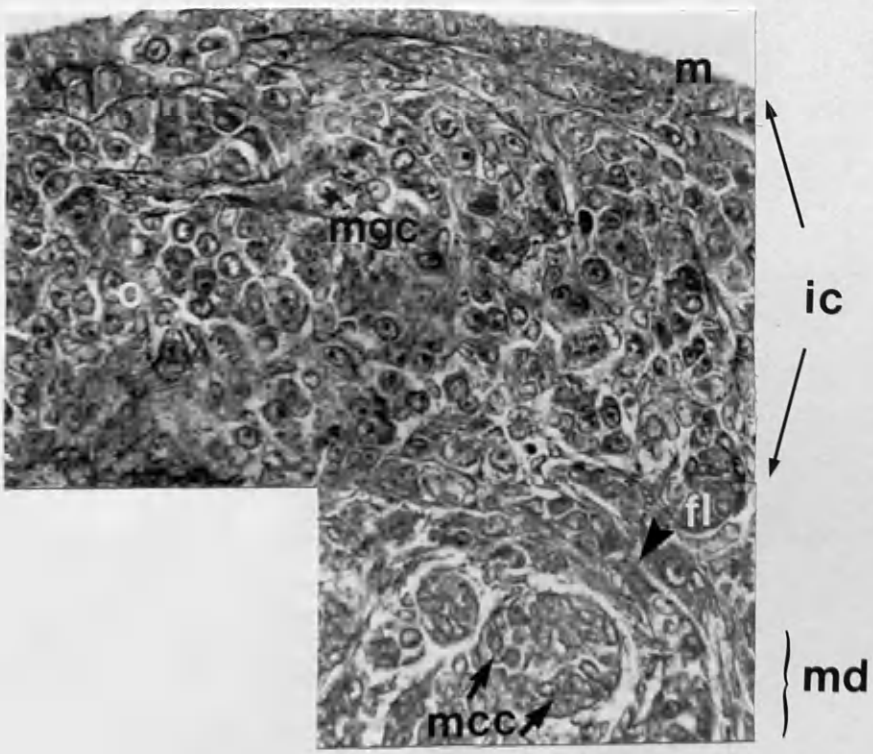


Fig. 3.27b

Fig. 3.28a. Longitudinal section through the ovary of a 14 day old pouch young bettong. (Bar = 100 μm).

Fig. 3.28b. Detail of Fig. 3.28a, showing the regular mesothelium (m), mitotic germ cells (mgc) forming oogonia and the differentiation of the blastema into small fibroblast-like cells (A) and large cells (B). (Bar = 50 μm).

Fig. 3.29a. Transverse section through the ovary of a 15 day old pouch young bettong. Note the development of the incipient cortex (c) and the medullary cords (mc). (Bar = 100 μm).

Fig. 3.29b. Detail of Fig. 3.29a. (Bar = 50 μm).

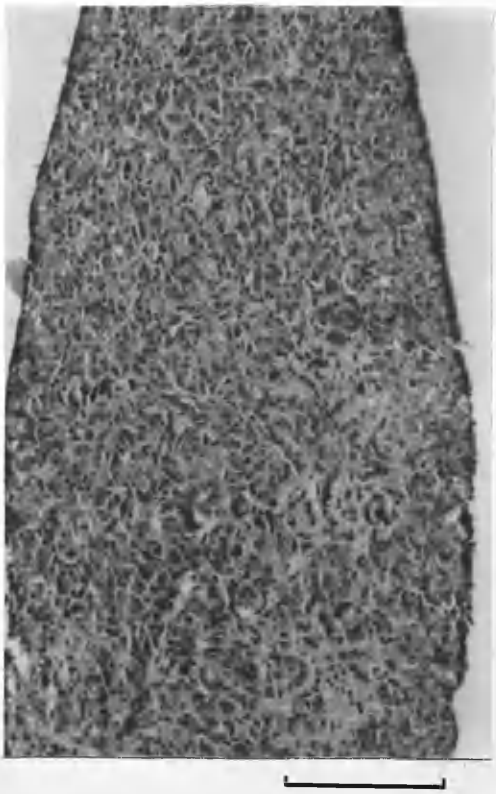


Fig. 3.28a

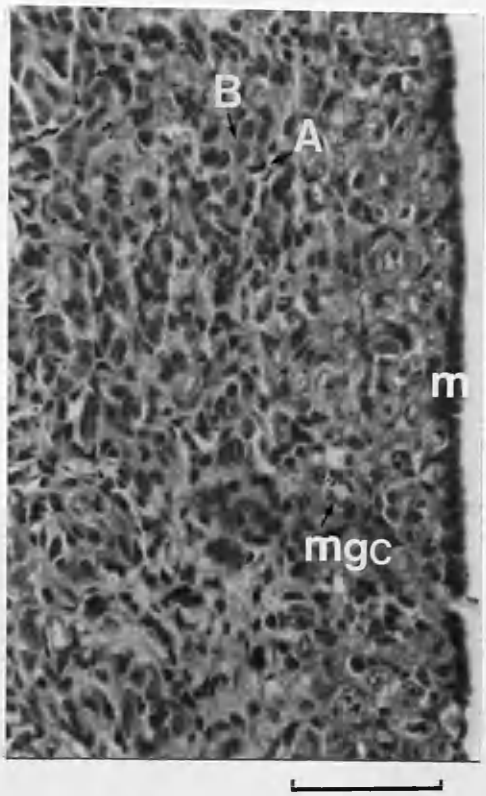


Fig. 3.28b

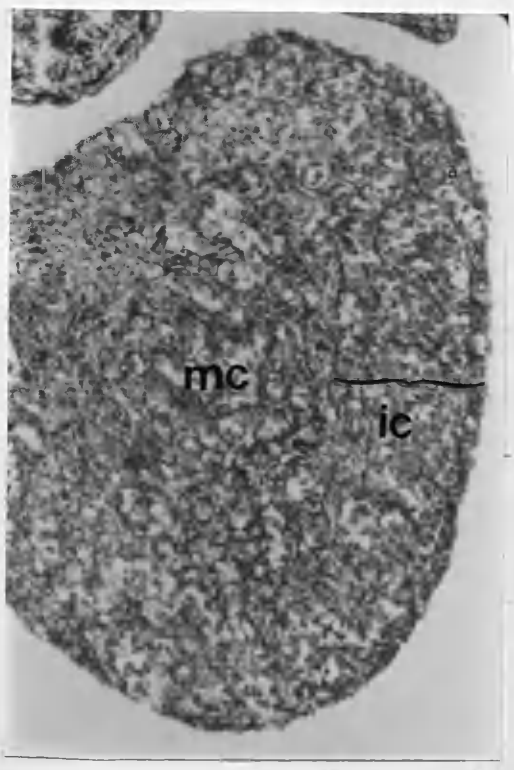


Fig. 3.29a

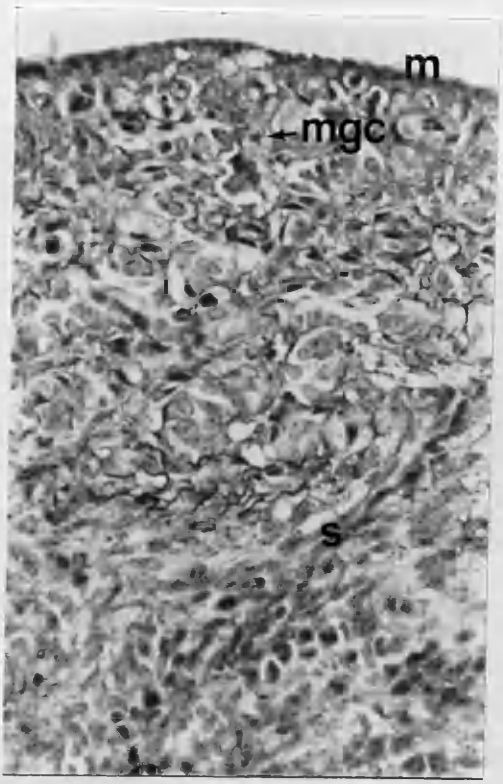


Fig. 3.29b

Fig. 3.30. Transverse section through the ovary of a 28 day old pouch young bettong. Note a thick layer (fl) separating the cortex (c) from the medulla (md). (Bar = 100 μm).

Fig. 3.31. Transverse section through the ovary of a 42 day old pouch young bettong to show the initiation of meiosis. (Bar = 50 μm).

Fig. 3.32. Transverse section through the ovary of a 42 day old pouch young bettong. Note the mesothelium (m), tunica albuginea (ta) and oocytes at different stages of meiosis. (Bar = 50 μm).

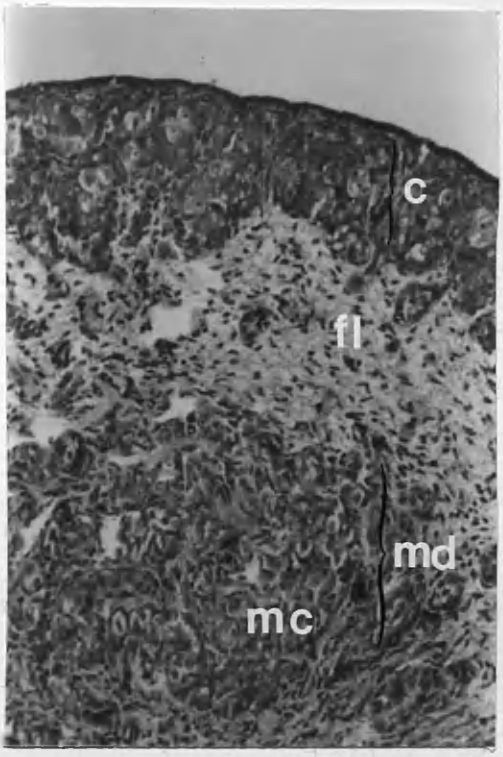


Fig. 3.30

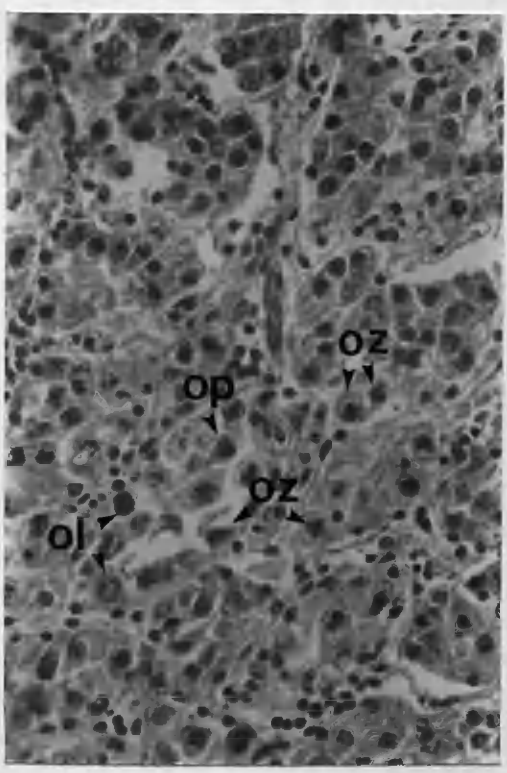


Fig. 3.31

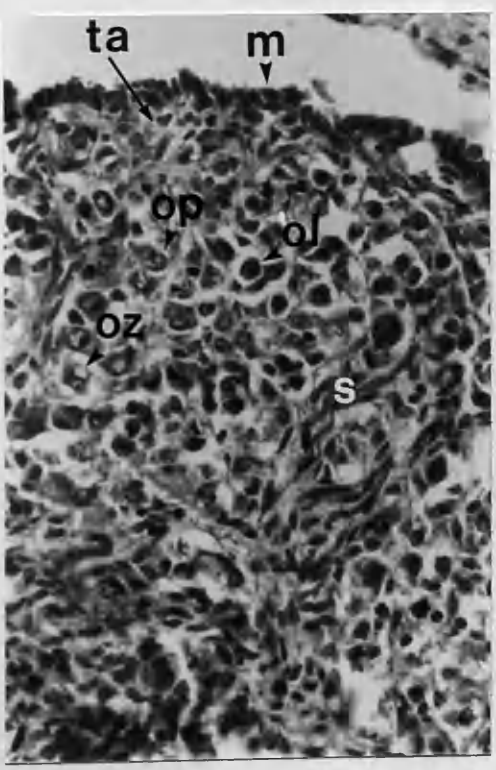


Fig. 3.32

Fig. 3.33. Transverse section through the ovary of a 56 day old pouch young bettong. Note the development of type 2 (2f) and type 3a (3af) follicles and the medullary cords (mc). (Bar = 100 μm).

Fig. 3.34. Transverse section through the ovary of a 59 day old pouch young bettong, showing medullary cords in the vicinity of type 3a (3af) follicle. (Bar = 100 μm).

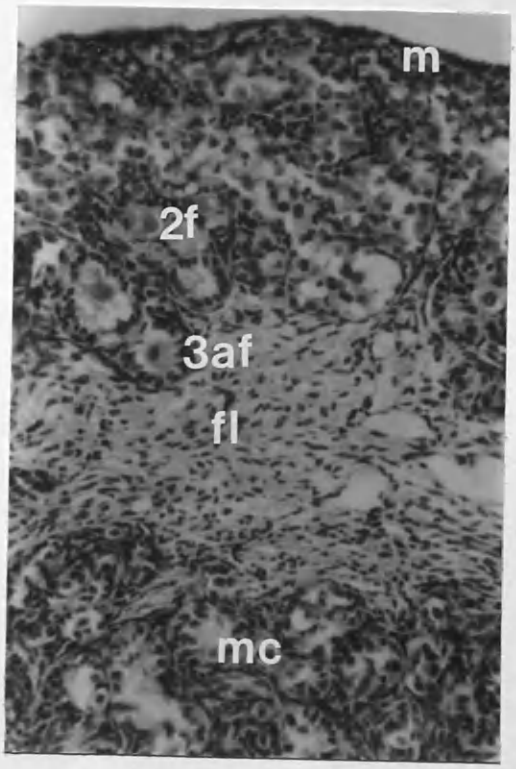


Fig. 3.33

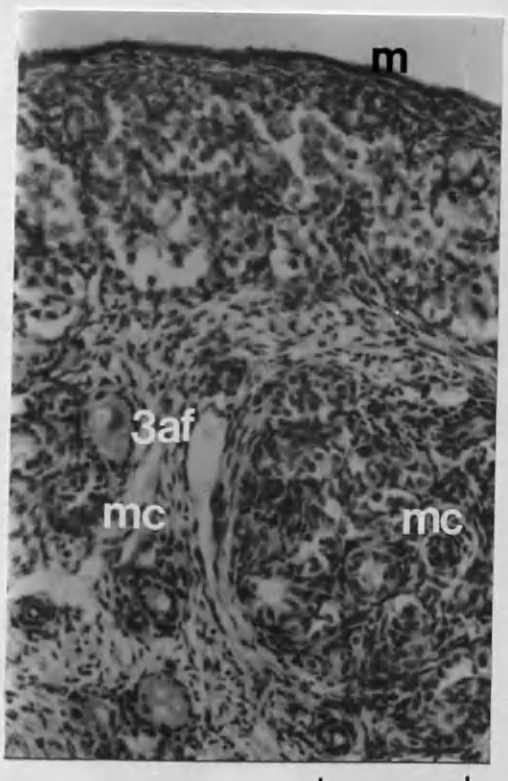


Fig. 3.34

Fig. 3.35a. Longitudinal section through the ovary and the mesonephros of an 8 day old potoroo. (Bar = 500 μm).

Fig. 3.35b. Detail of Fig. 3.35a to show the rete (r) at the hilar region. (Bar = 50 μm).

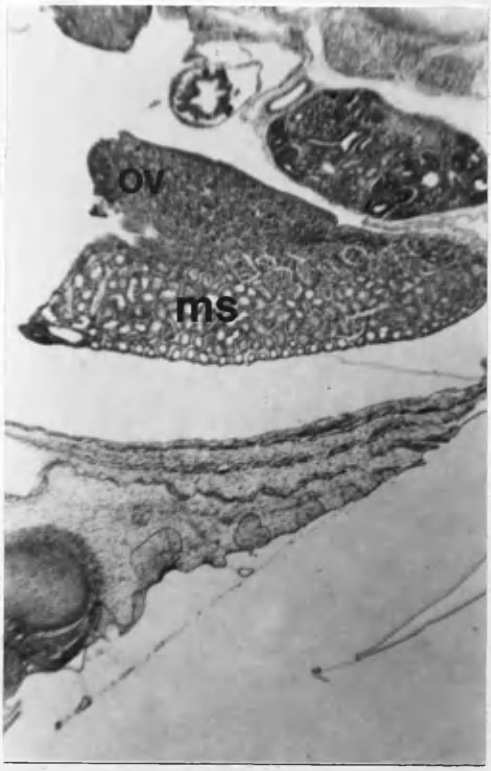


Fig. 3.35 a

Fig. 3.35 b

Fig. 3.36a. Longitudinal section through the ovary of a 15 day old pouch young potoroo. (Bar = 100 μm).

Fig. 3.36b. Detail of marked region in Fig. 3.36a. Note mitotic germ cells giving rise to oogonia. (Bar = 25 μm).

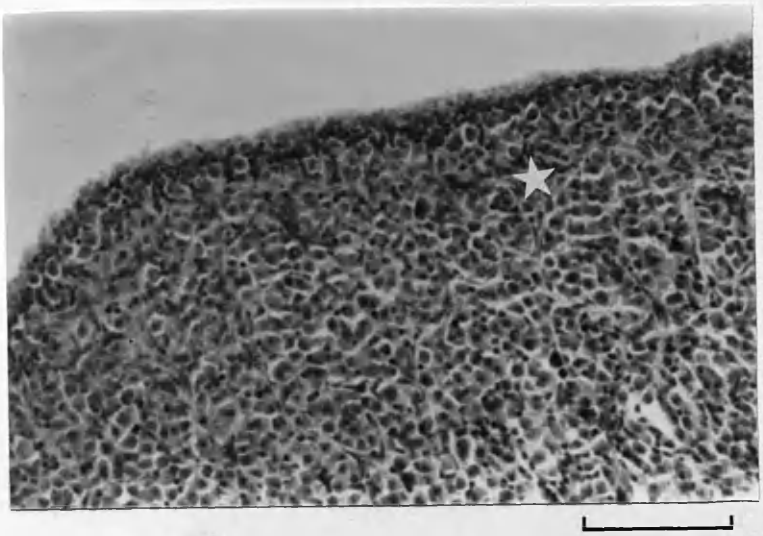


Fig. 3.36a

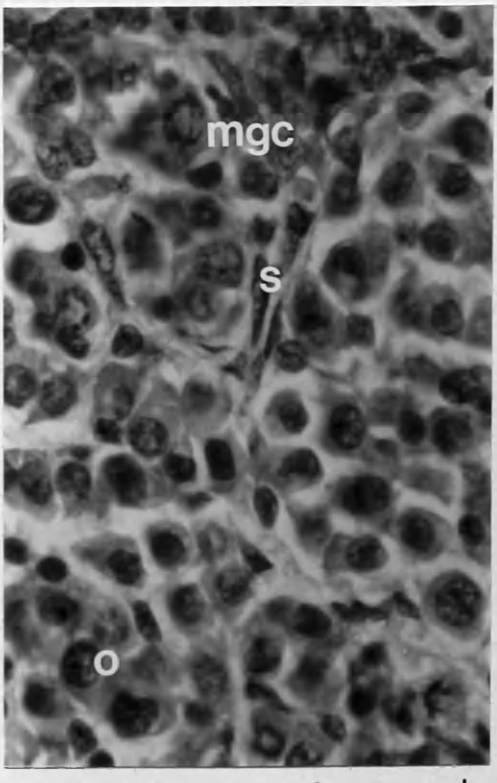


Fig. 3.36b

Fig. 3.37a. Longitudinal section through the ovary of a 32 day old pouch young potoroo. Note the migration of oogonia to the periphery forming the incipient cortex (ic). (Bar = 100 μm).

Fig. 3.37b. Detail of the cortex (c) from Fig. 3.37a showing mitotic oogonia (o). (Bar = 25 μm).

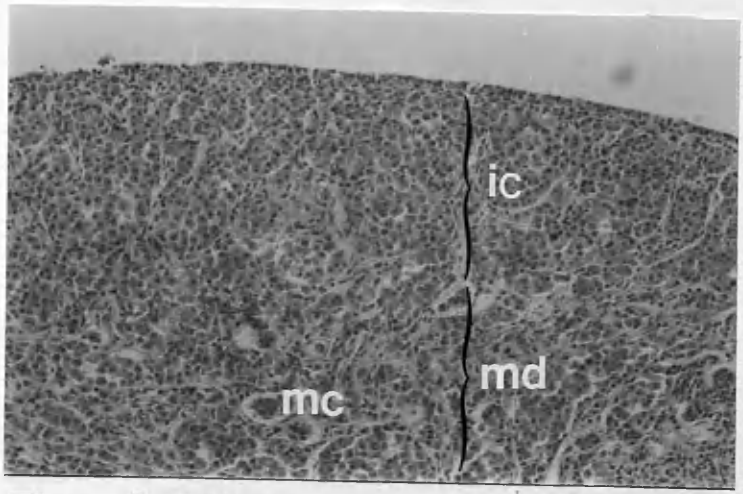


Fig. 3·37a

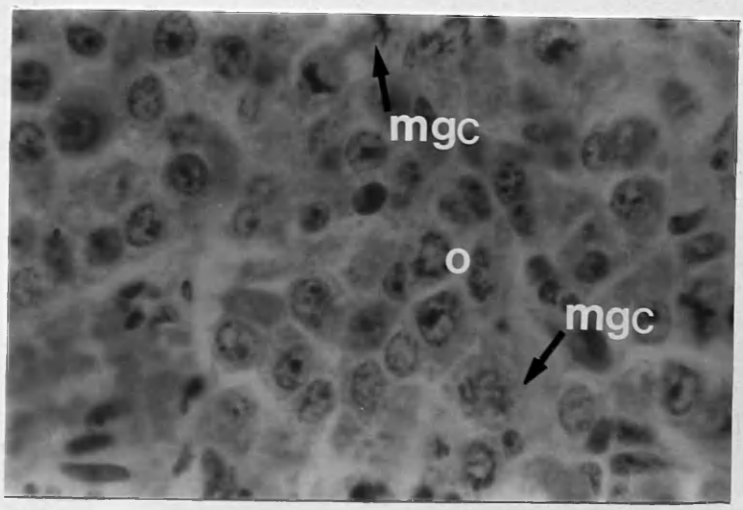


Fig. 3·37b

Fig. 3.38a. Transverse section through the ovary of a 46 day old pouch young potoroo. Note the presence of a layer of fibroblast cells (fl) separating the cortex (c) from the medulla (md). (Bar = 100 μm).

Fig. 3.38b. Detail of marked region from Fig. 3.38a. (Bar = 25 μm).

Fig. 3.39. Longitudinal section through the ovary of a 48 day old pouch young potoroo showing the development of the medullary cord (mc) and the rete (r) in the hilar region (h). (Bar = 100 μm).

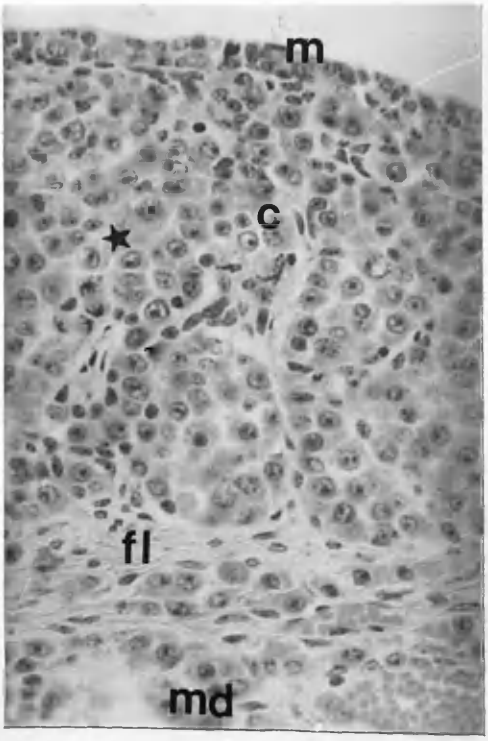


Fig. 3.38a

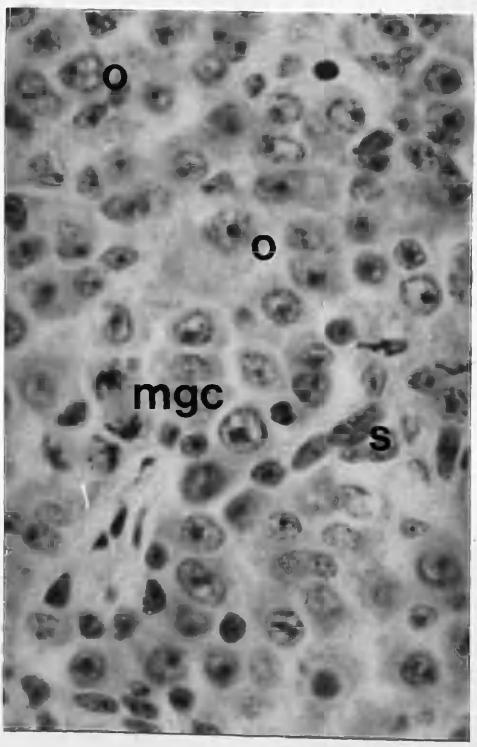


Fig. 3.38 b

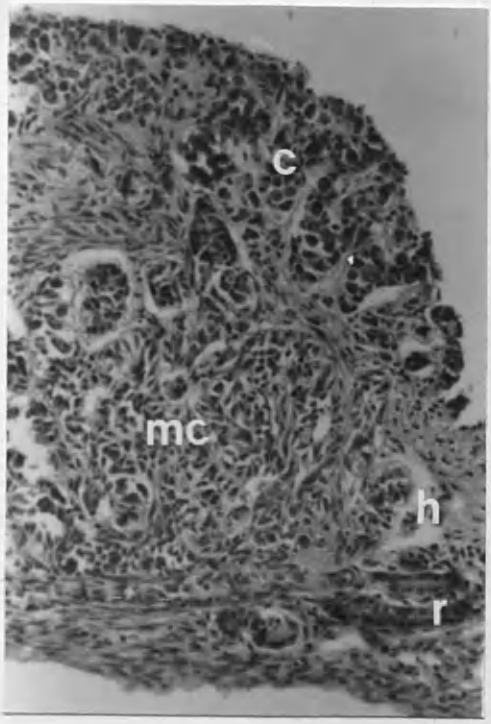


Fig. 3.39

Fig. 3.40a. Transverse section through the ovary of 50 day old pouch young potoroo. (Bar = 100 μm).

Fig. 3.40b. Detail of Fig. 3.40a. Meiosis has commenced in the germ cell nests which are closest to the medulla: leptotene (ol), zygotene (oz) and pachytene stages (op) are present, though oogonia (o) are still found. (Bar = 50 μm).

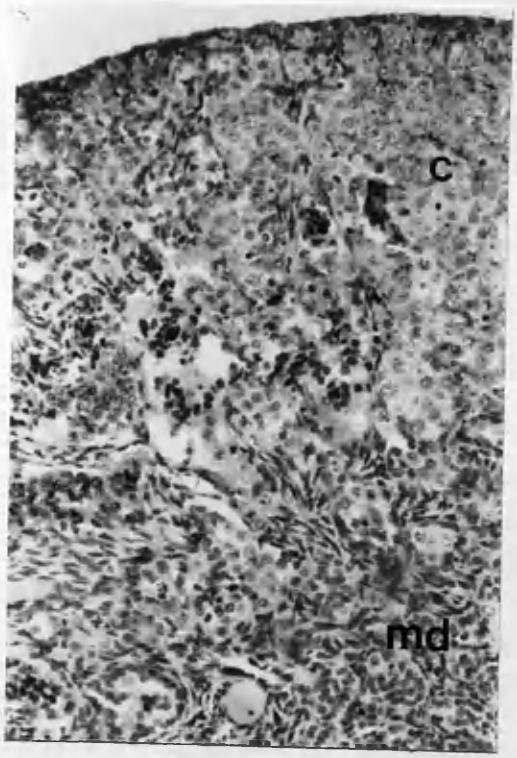


Fig. 3.40a

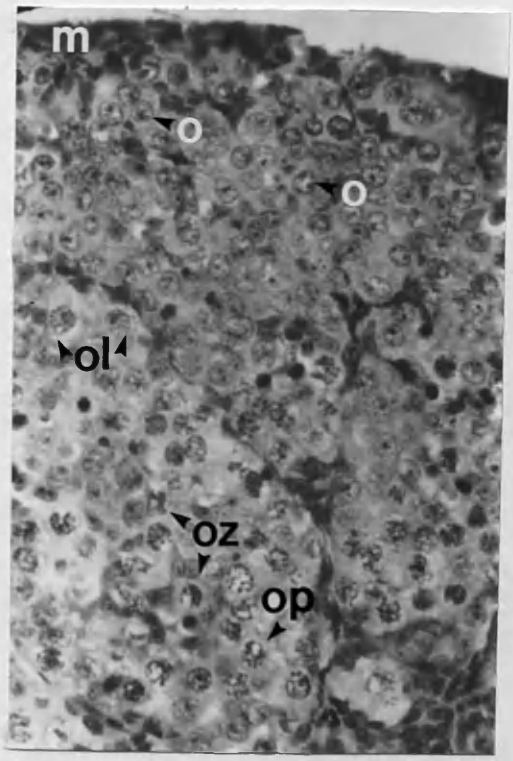


Fig. 3.40b

Fig. 3.41. Transverse section through the ovary of a 51 day old pouch young potoroo . Note that the majority of germ cells are at meiotic prophase. (Bar = 25 μm).

Fig. 3.42a. Transverse section through the ovary of a 65 day old pouch young potoroo. Note well developed medullary cords (mc). (Bar = 100 μm).

Fig. 3.42b. Detail of Fig. 3.42a to show the irregularly shaped medullary cord cells (mcc). (Bar = 50 μm).

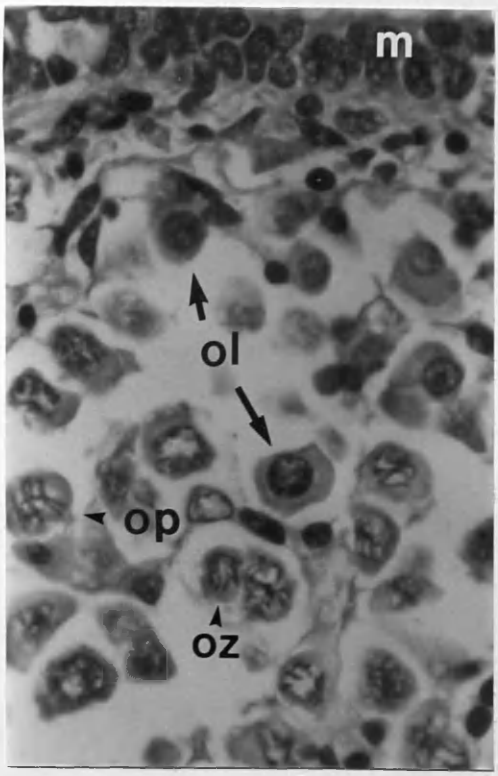


Fig. 3·41

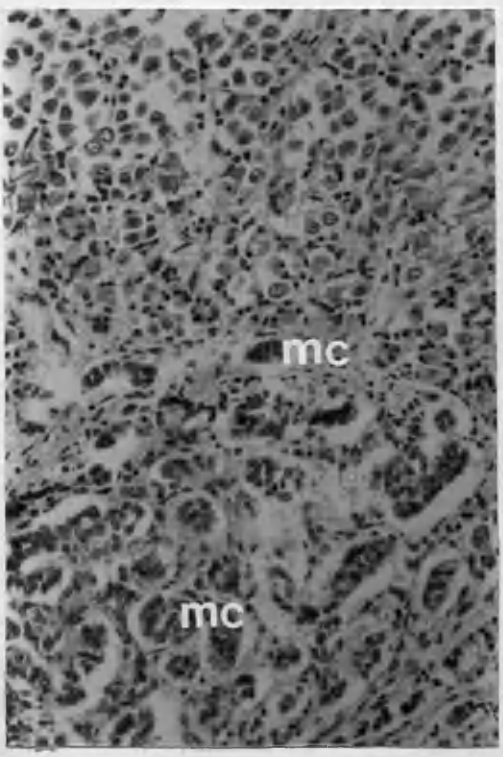


Fig. 3·42a

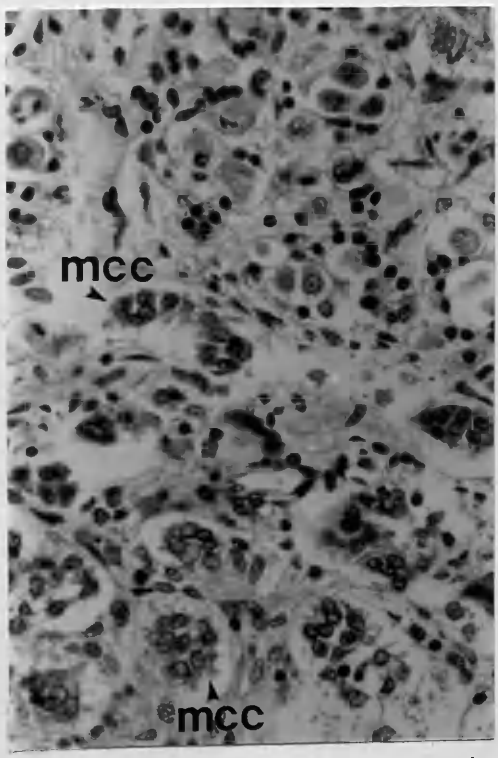


Fig. 3·42b

Fig. 3.43a. Transverse section through the ovary of a 66-69 day old pouch young potoroo (chronological age 86 days). a) Note the development of type 2 follicles (2f) and the tunica albuginea (ta). (Bar = 50 μm).

Fig. 3.43b. Electron micrograph of the oocytes from the same ovary as that shown in Fig. 3.43a. Note the nucleus (n), nucleolus (nu), mitochondria (mi), Golgi complex (G), rough endoplasmic reticulum (er) and polyribosomes (pr). x 6 K.

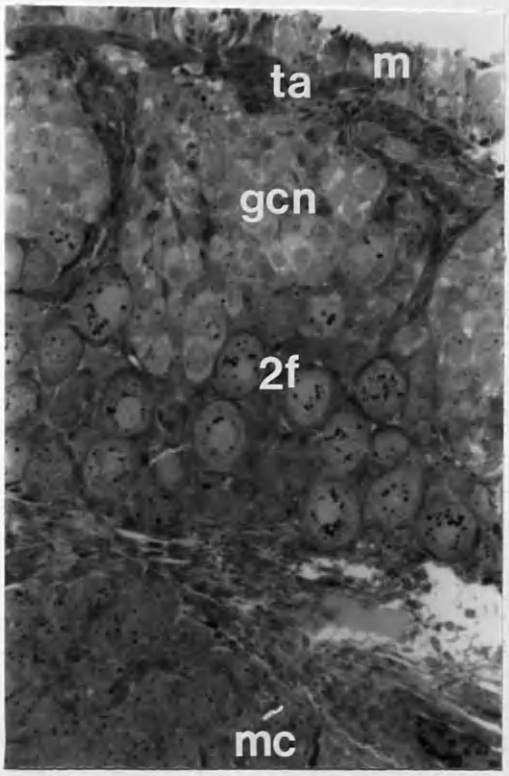


Fig. 3.43a

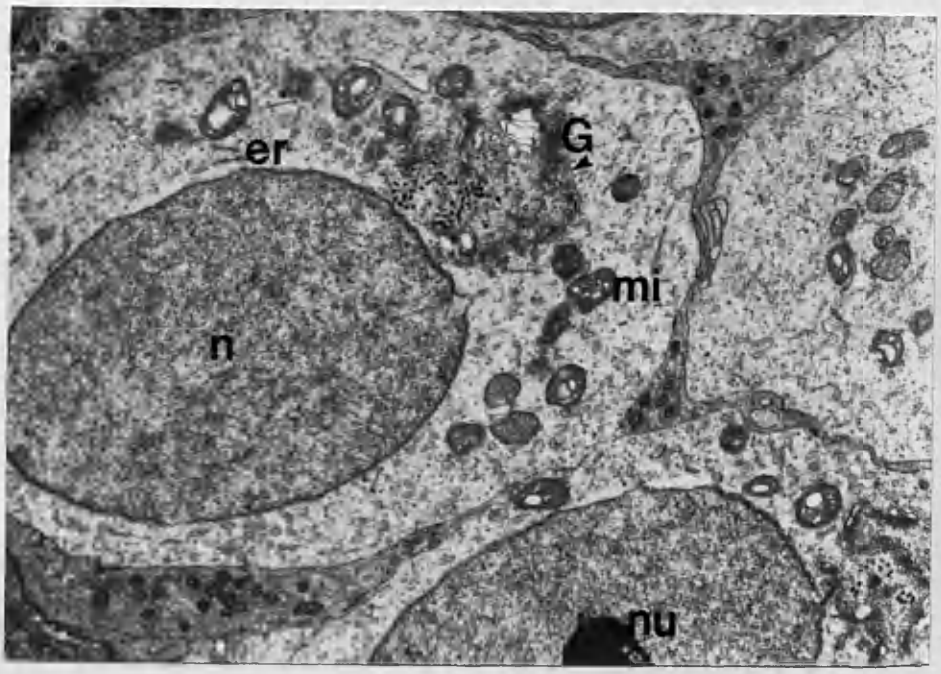


Fig. 3.43b

Fig. 3.43c. Note the medullary cords (mc). (Bar = 50 μm).

Fig. 3.43d. Electron micrograph. Note the medullary cords (mc) clumping together. The basal lamina is lacking at the point of contact between the cords. x 2.4 K.

Fig. 3.43e. Detail of marked region in 3.43d showing the structure of a medullary cord cell (mcc). Note the mitochondria (mi) and lipid triplets (L). x 8.82 K.

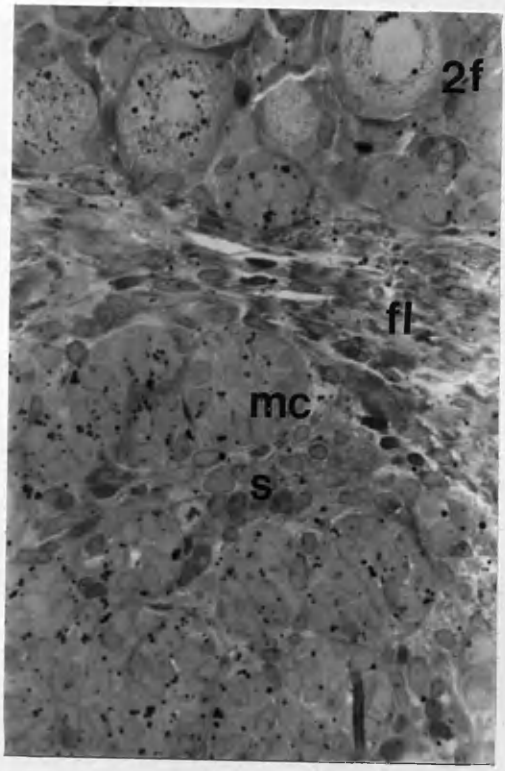


Fig. 3.43c

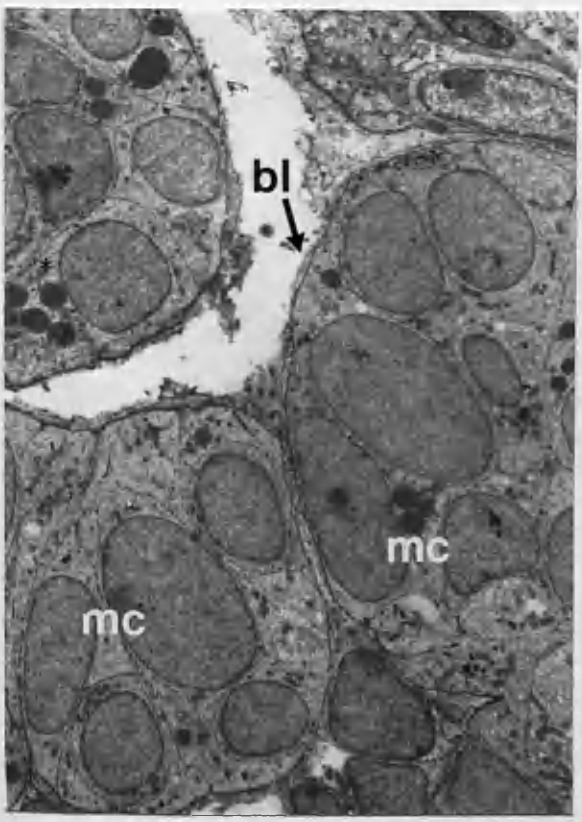


Fig. 3.43d

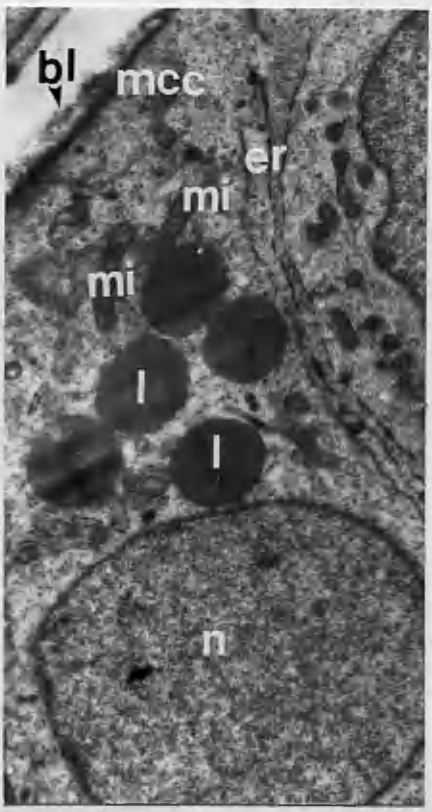
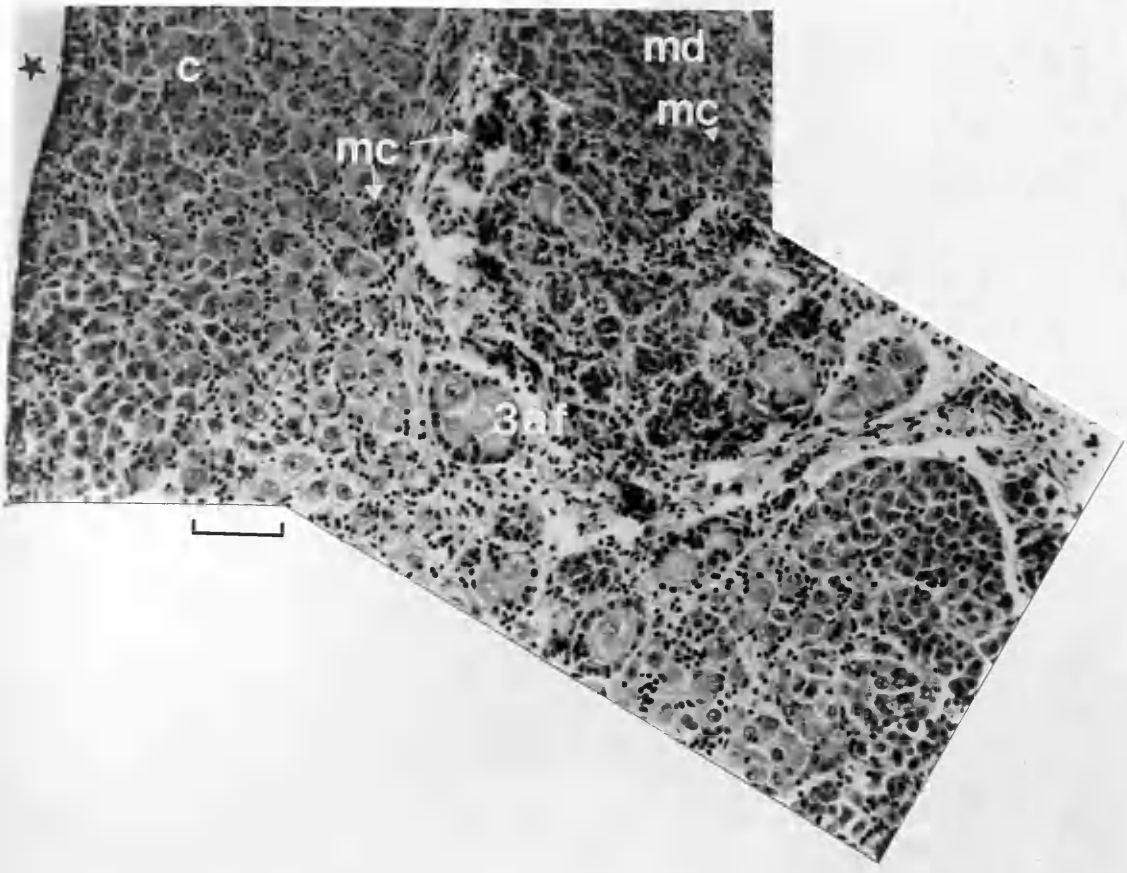


Fig. 3.43e

Fig. 3.44a. Transverse section through the ovary of a 70 day old pouch young potoroo. The medullary cords (mc) penetrate the fibrous layer (fl) which separates the cortex (c) from the medulla (md). Some of these cords are found close to type 3a follicles (3af). (Bar = 25 μm).

Fig. 3.44b. Detail of marked region in 3.44a to show the cortex (c) with oogonia (o), binucleate oocyte (bo) and oocytes at leptotene (ol) and zygotene (oz) stages. (Bar = 25 μm).

Fig. 3.44a



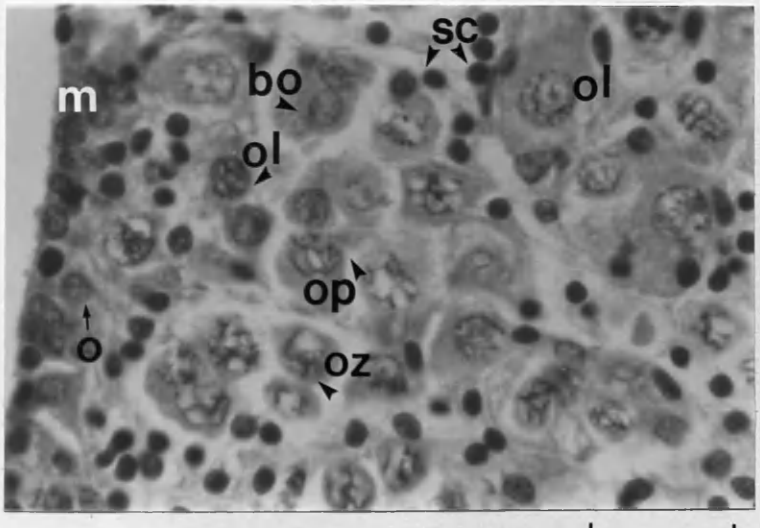


Fig. 3.44b

Fig. 3.45a. Transverse section through the ovary of a 75 day old potoroo. a) Note oocytes at leptotene (ol), zygotene (oz), pachytene (op) and diplotene (od) stages. (Bar = 50 μm).

Fig. 3.45b. The medullary cords (mc) are dissociating to give rise to granulosa cells. (Bar = 25 μm).

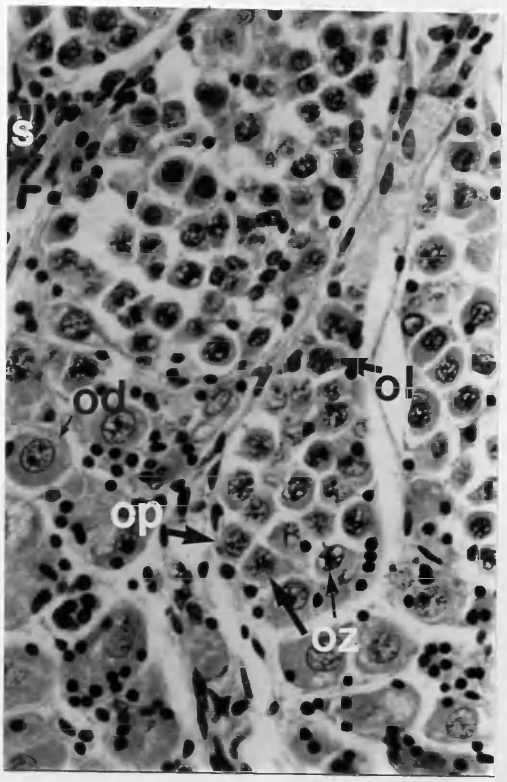


Fig. 3.45a

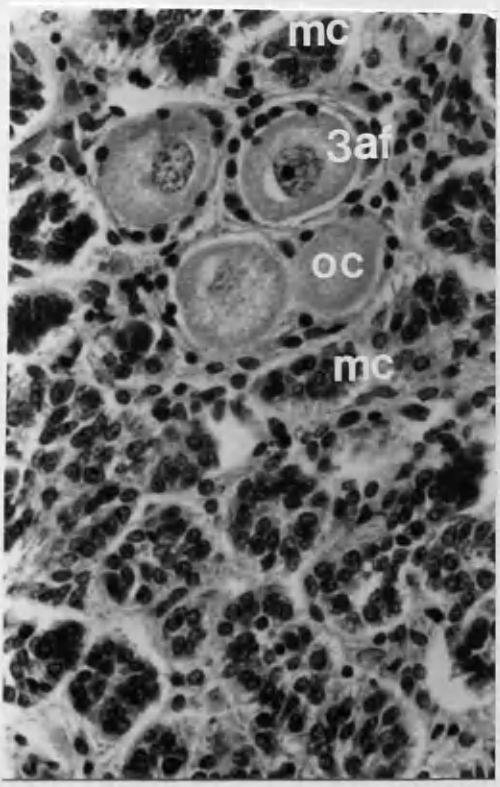


Fig. 3.45b

Fig. 3.46a. Transverse section through the ovary of an 82 day old pouch young potoroo. a) Note the development of types 2 (2f), 3a (3af) and 3b (3bf) follicles and a type 3b follicles with 4 oocytes. (Bar = 100 µm).

Fig. 3.46b. Note a type 4 follicle (4f) with 20 oocytes. (Bar = 100 µm).

Fig. 3.46c. Note the type 3b (3bf) biovular follicle and the medullary cords (mc). (Bar = 50 um). (Bar = 50 µm).

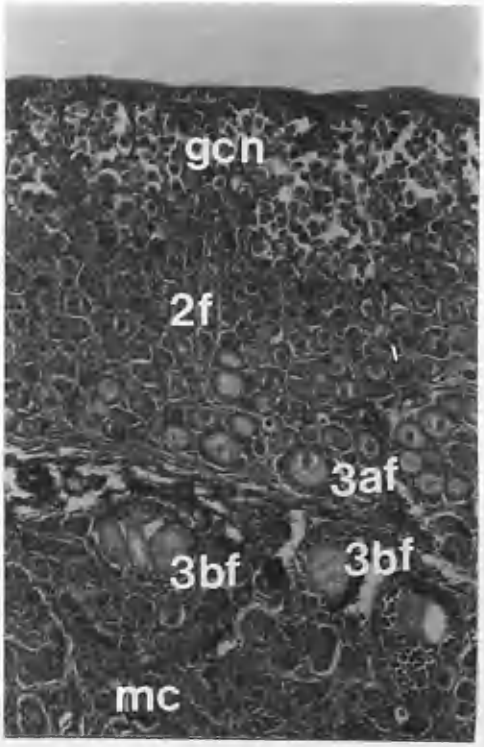


Fig. 3.46a

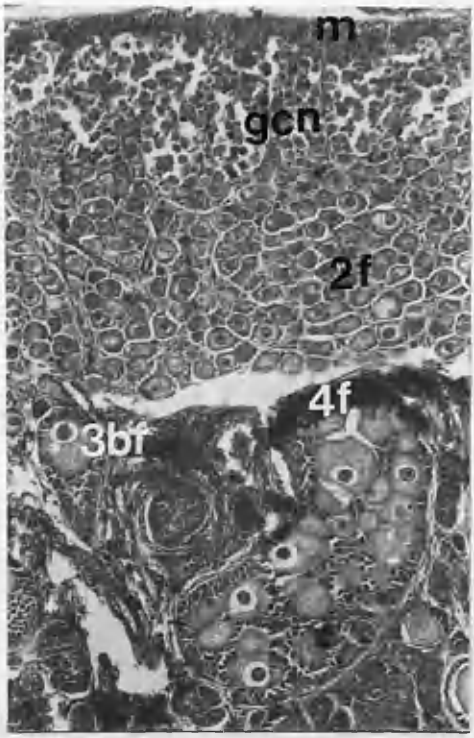


Fig. 3.46b

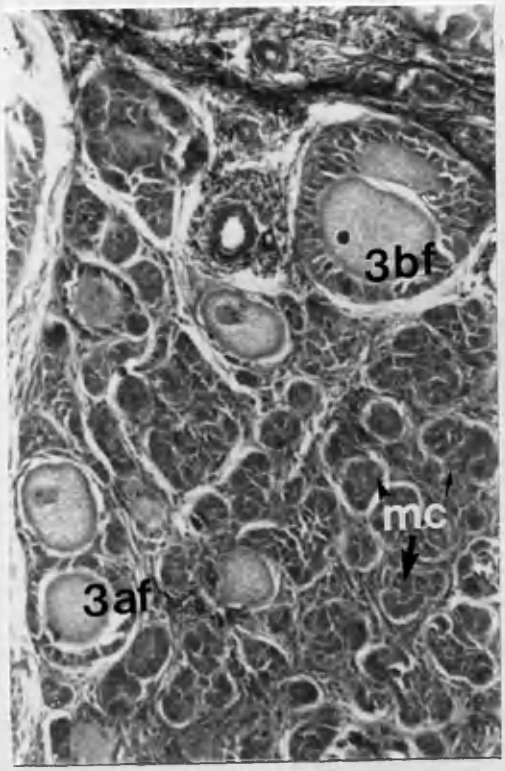


Fig. 3.46c

Fig. 3.46d. Note triovular follicles of types 3a (3af) and 3b (3bf) with oocytes which are in close contact with each other. (Bar = 100 μm).

Fig. 3.46e. Note the medullary cord (mc) which is continuous with the granulosa layer (gl). (Bar = 25 μm).

Fig. 3.46f. Note the difference between the solid medullary cords and the patent rete tubules. (Bar = 50 μm).

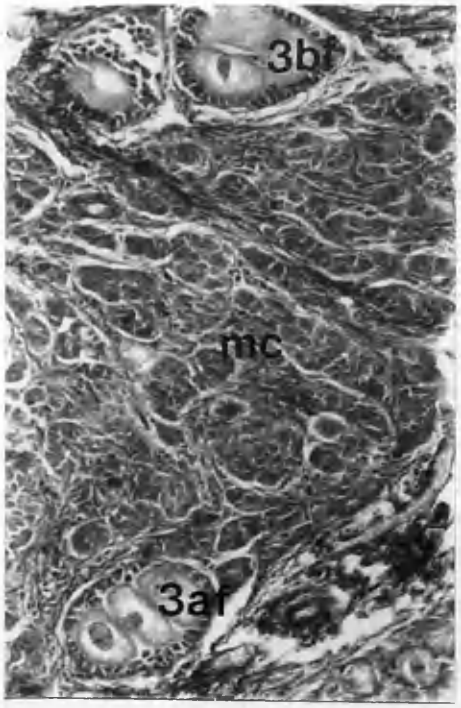


Fig 3.46d

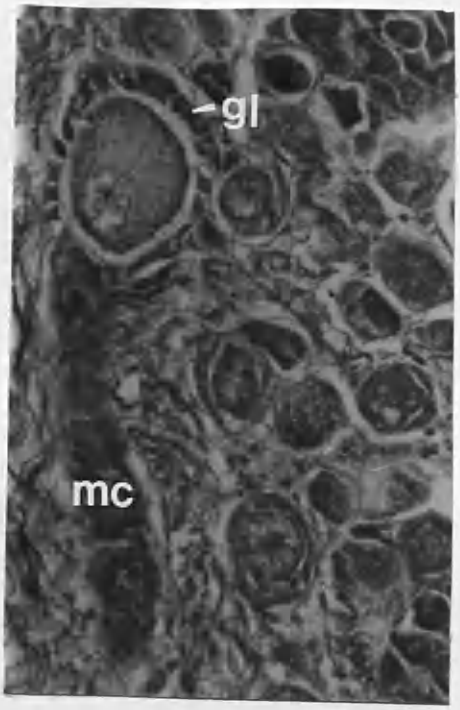


Fig. 3.46e

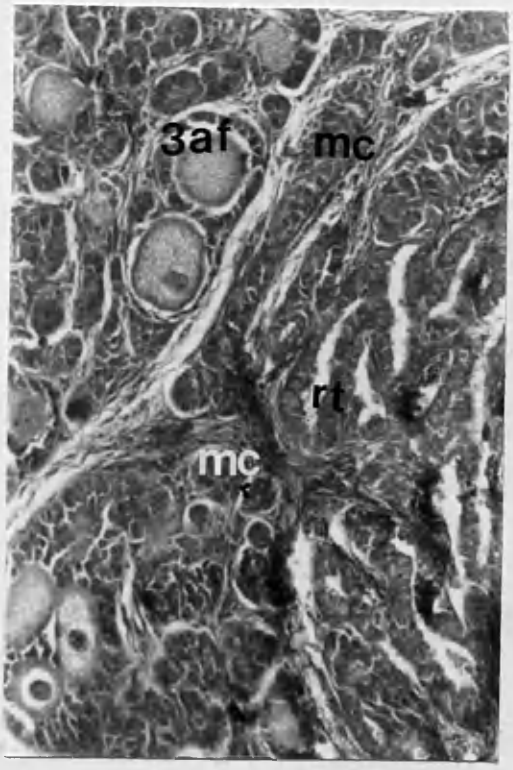


Fig. 3.46f

Fig. 3.47a. Transverse section through the ovary of a 91 day old pouch young potoroo. Note different types of follicles and some biovular type 3b follicles (3bf). (Bar = 100 μm).

Fig. 3.47b. Detail of marked region of Fig. 3.47a to show the mesothelium (m) tunica albuginea (ta) and germ cell nests (gcn) with oocytes at different stages of meiosis. (Bar = 50 μm).

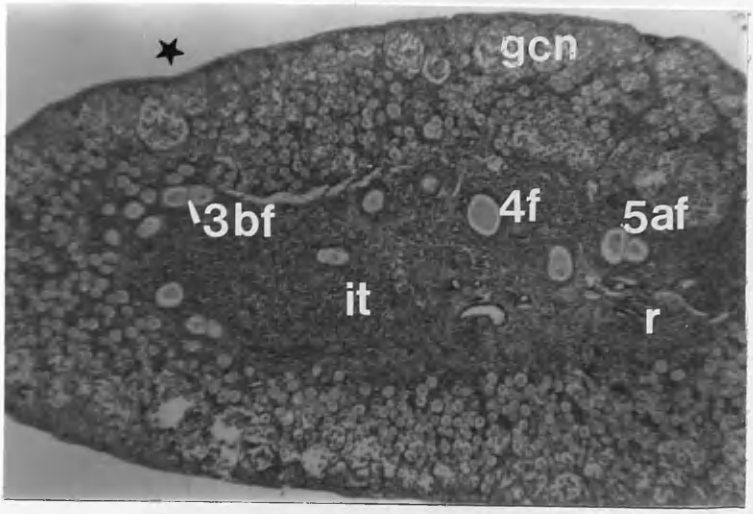


Fig. 3.47a

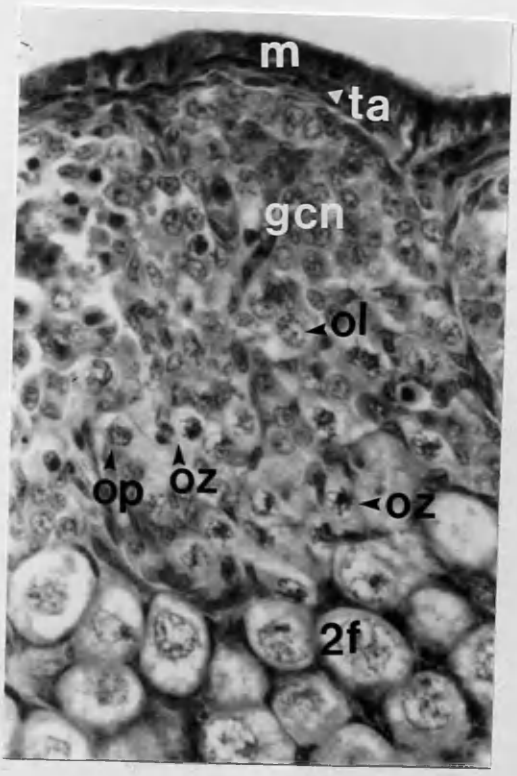


Fig. 3.47b

Fig. 3.48a. Transverse section through the ovary of a 92 day old pouch young potoroo. Note the development of the interstitial tissue (it). (Bar = 100 um).

Fig. 3.48b. Detail of the cortex. (Bar = 50 um).

Fig. 3.48c. Detail of the medulla showing medullary cords (mc) giving rise to granulosa cells and clusters of interstitial tissue (it). (Bar = 50 um).

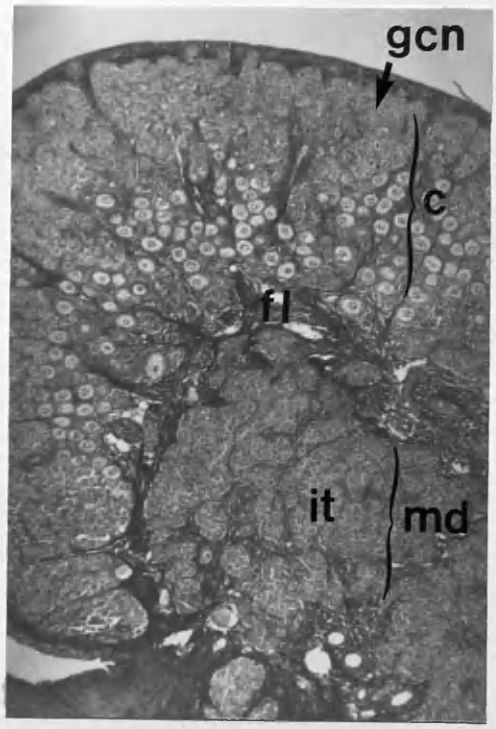


Fig. 3·48a

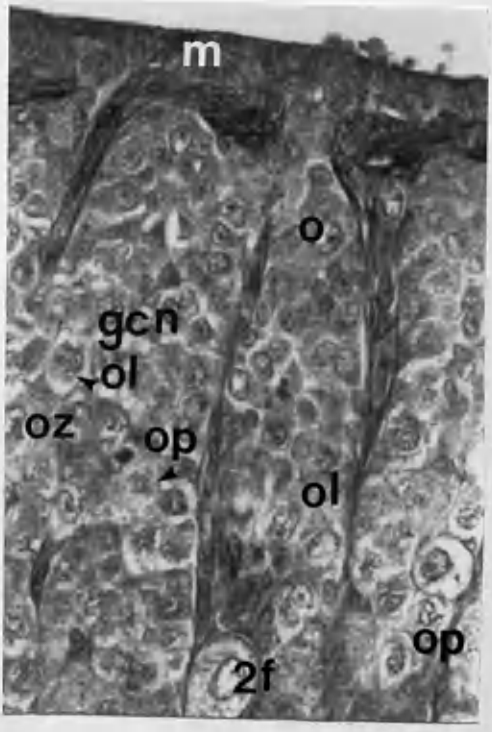


Fig. 3·48b

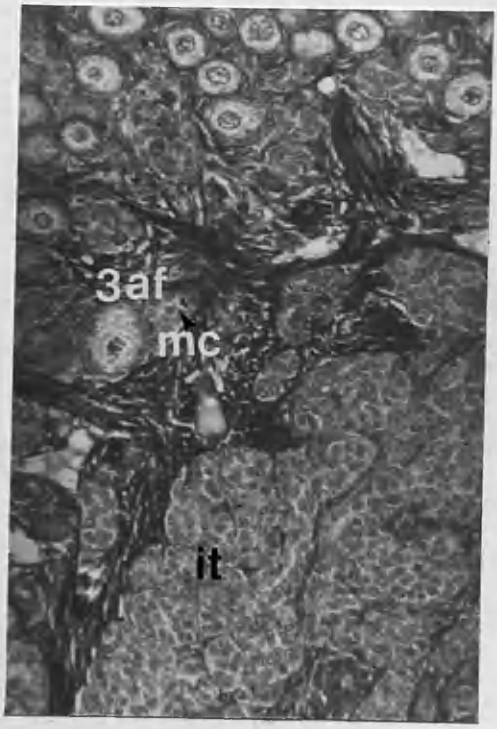


Fig. 3·48c

Fig. 3.49a. Transverse section through the ovary of a 92 day old pouch young potoroo. a) Note the cortical zonation; the rete (r) localized in the anterior region; the interstitial tissue (it) and numerous polyovular follicles (pof). (Bar = 500 μm).

Fig. 3.49b. Detail of Fig. 3.49a to show the cortex with oocytes at different stages of meiosis. (Bar = 50 μm).

Fig. 3.49c. Note the presence of follicles of type 5a (5af). (Bar = 50 μm).

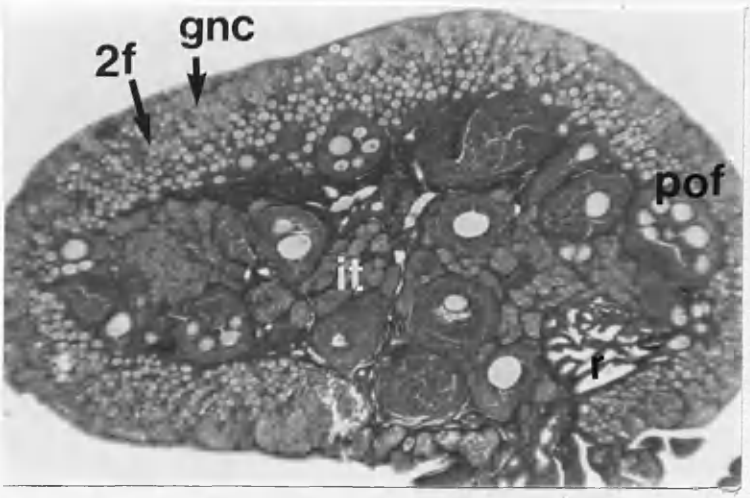


Fig. 3·49a

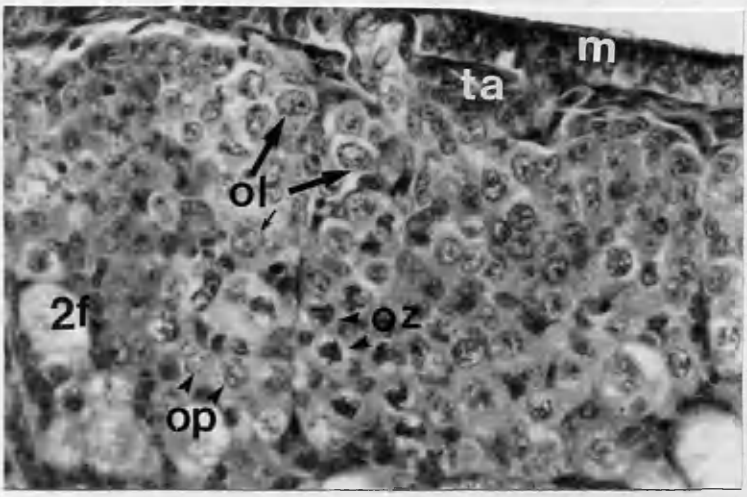


Fig. 3·49b

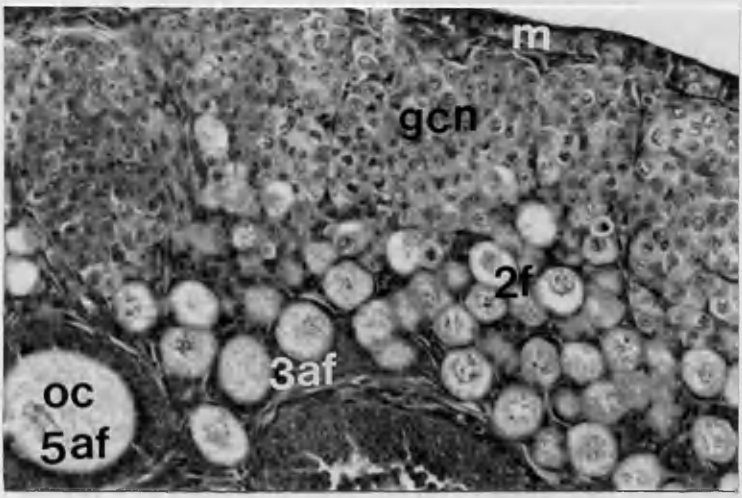


Fig. 3·49c

Fig. 3.49d. Note a biovular follicles of type 5b (5bf) with oocytes separated from each other by granulosa cells (g) and a biovular follicle of type 7 (7f) with oocytes in close contact with each other. (Bar = 100 μm).

Fig. 3.49e. Type 6 follicle (6f) with 5 oocytes. (Bar = 100 μm).

Fig. 3.49f. Type 6 follicle (6f) with 3 oocytes. (Bar = 100 μm).

Fig. 3.49g. Type 6 follicle (6f) with 4 oocytes. (Bar = 100 μm).

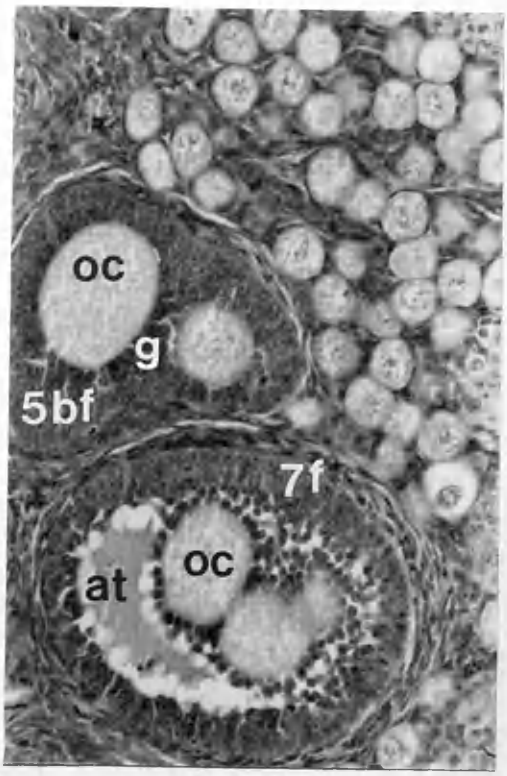


Fig. 3.49d

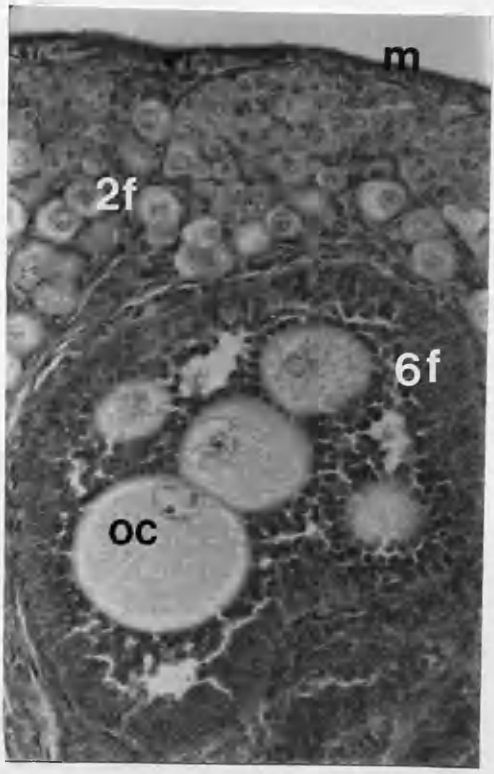


Fig. 3.49e

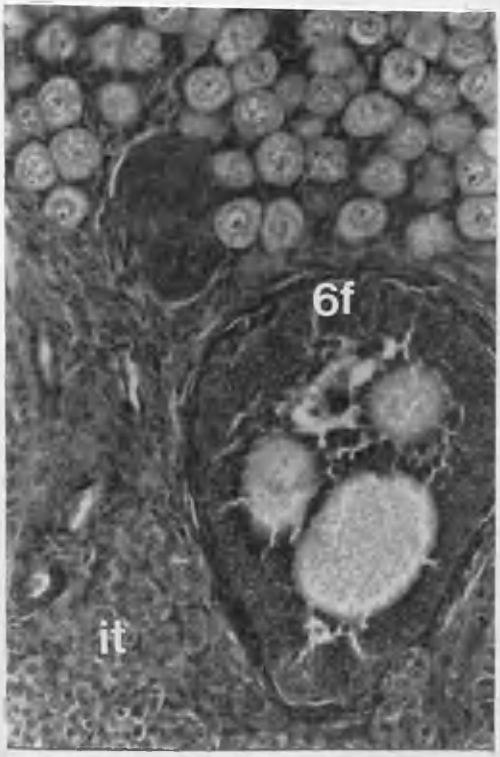


Fig. 3.49f

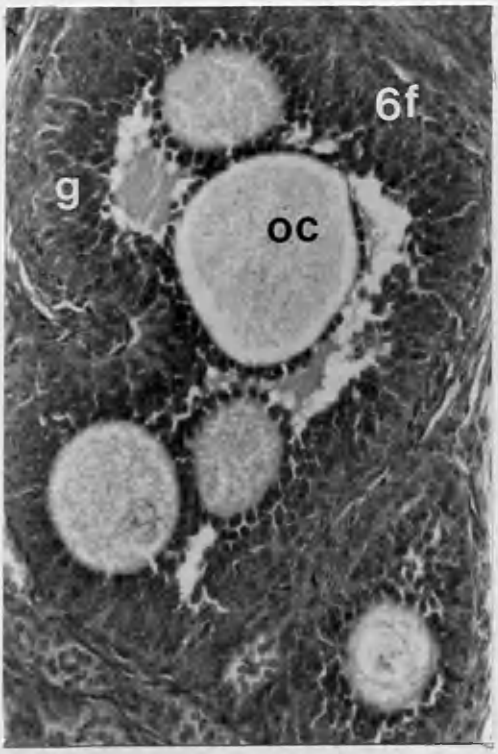


Fig. 3.49g

Fig. 3.49h. Type 6 follicle (6f) with 6 oocytes, 2 of which are in close contact with each other. (Bar = 100 μm).

Fig. 3.49i. Type 5b follicle (5bf) with 4 oocytes and type 7 follicle (7f). (Bar = 100 μm).

Fig. 3.49j. Type 5a follicle (5af) with 2 oocytes and a type 5b follicle (5bf) with 9 oocytes. (Bar = 100 μm).

Fig. 3.49k. Type 4 follicle (4f) with 3 oocytes and a type 6 follicle (6f) with 3 oocytes. (Bar = 100 μm).

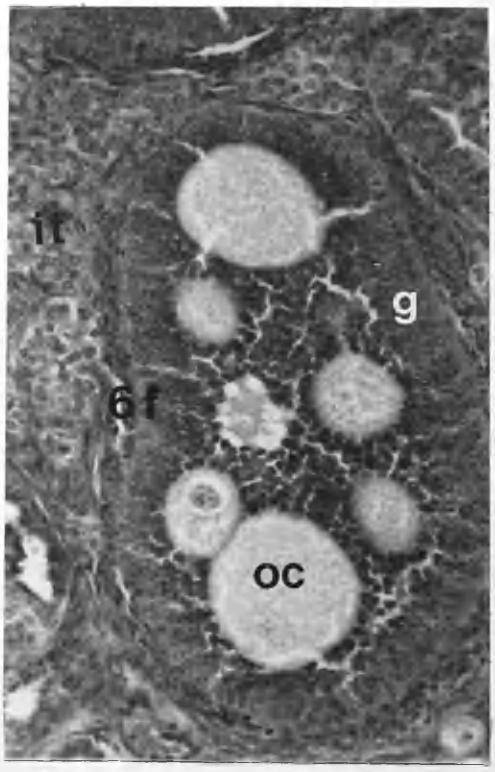


Fig. 3.49 h

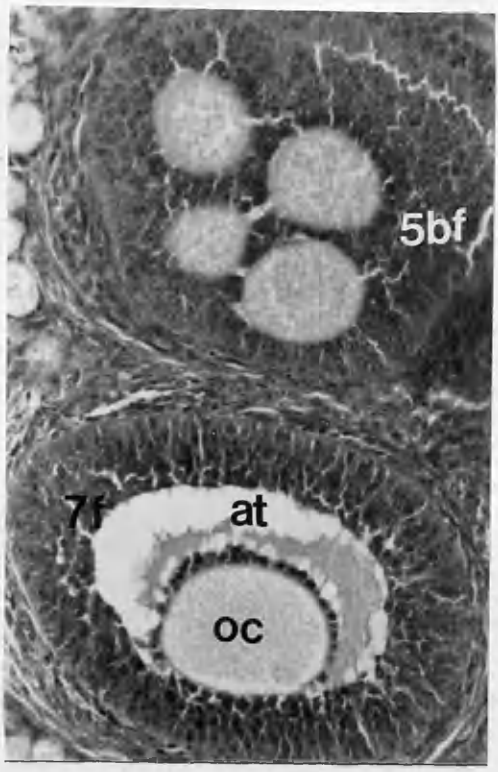


Fig. 3.49 i

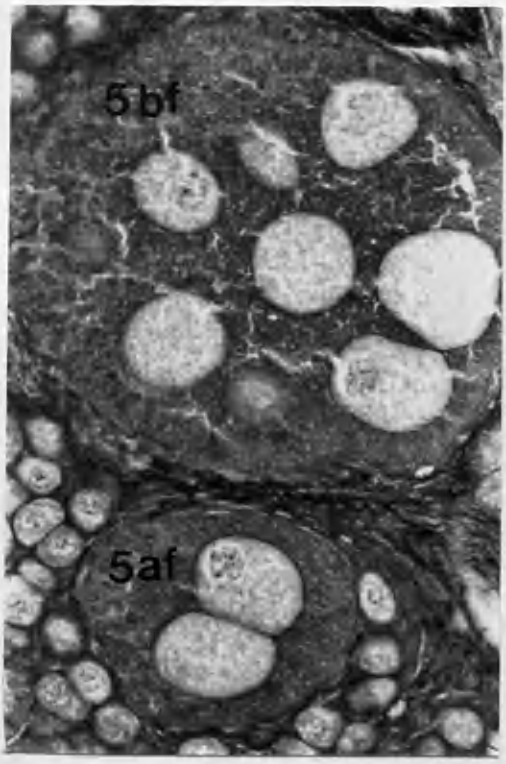


Fig. 3.49 j

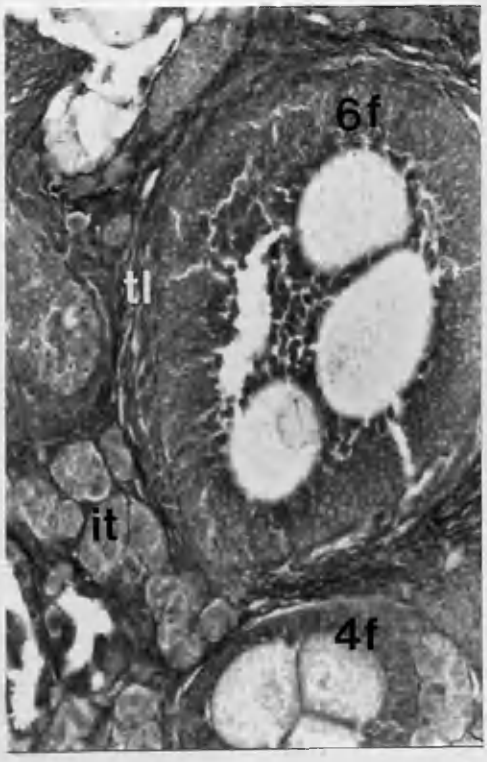


Fig. 3.49 k

Fig. 3.49l. Note interstitial tissue clusters (it) and a biovular follicle of type 5b (5bf). (Bar = 50 μm).

Fig. 3.49m. Note medullary cords (mc) and rete tubules (rt). (Bar = 50 μm).

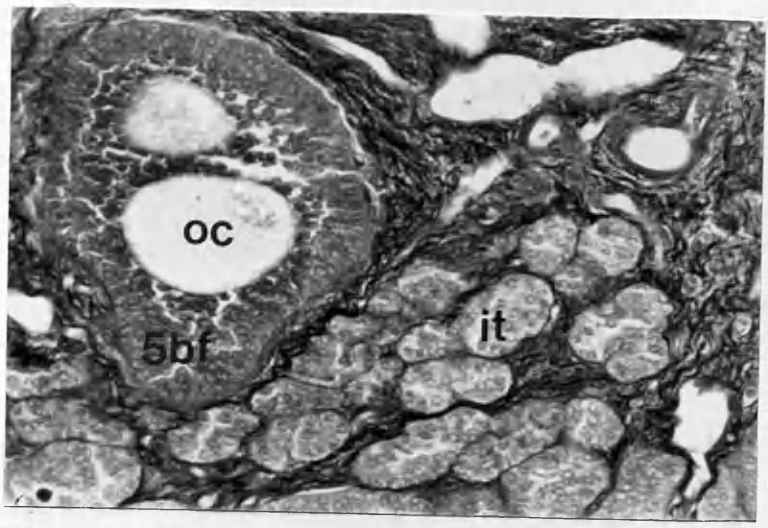


Fig. 3.49l

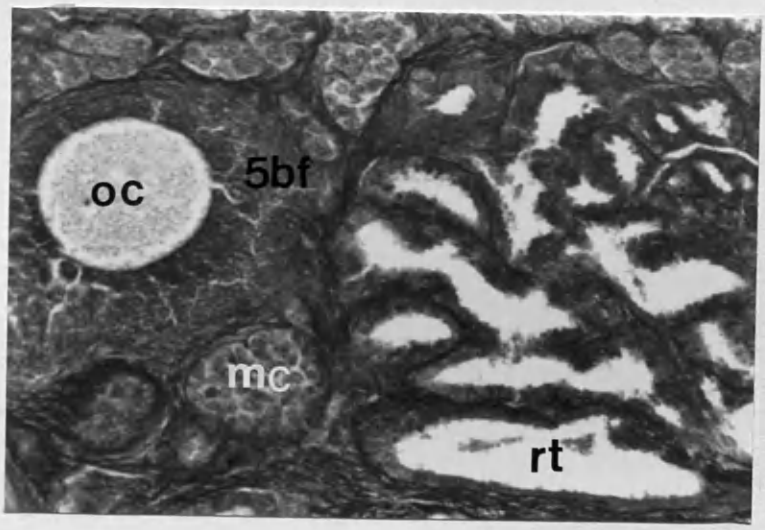


Fig. 3.49m

Fig. 3.50a. Transverse section through the ovary of a 98 day old potoroo. a) Note the rete (r) at the hilar region. (Bar = 500 μm).

Fig. 3.50b. Detail of the cortex, showing atretic oocytes (ao). (Bar = 50 μm).

Fig. 3.50c. Detail of the medulla. Note atretic follicle (af). (Bar = 50 μm).

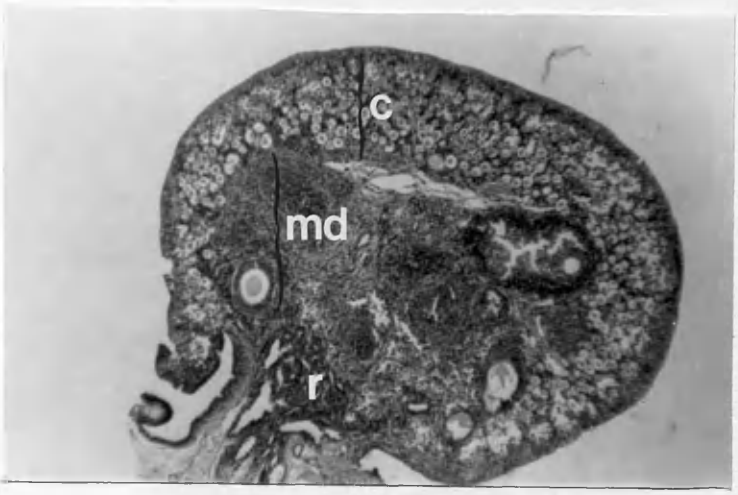


Fig. 3.50a

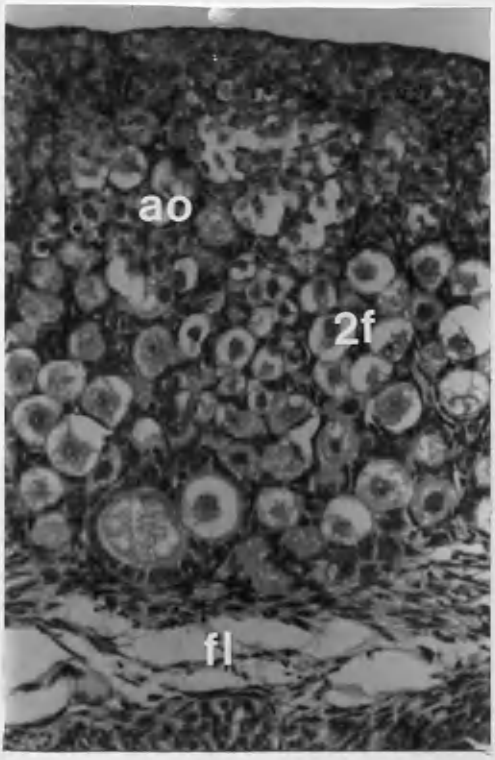


Fig. 3.50b

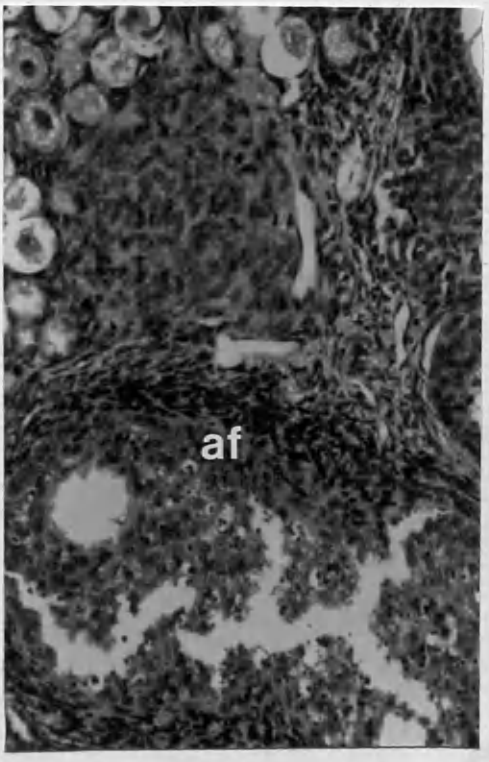


Fig. 3.50c

Fig. 3.50d. Degenerating biovular follicle (af). (Bar = 100 µm).

Fig. 3.50e. A healthy type 5a follicle (5af) with 5 oocytes. (Bar = 100 µm).

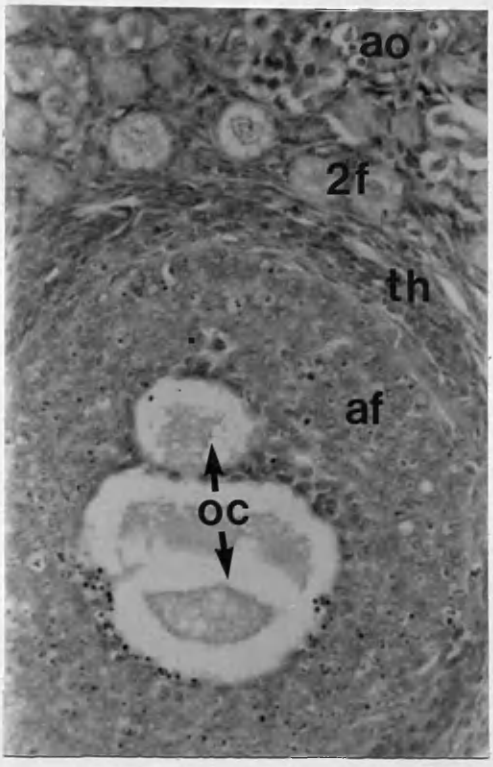


Fig. 3.50 d

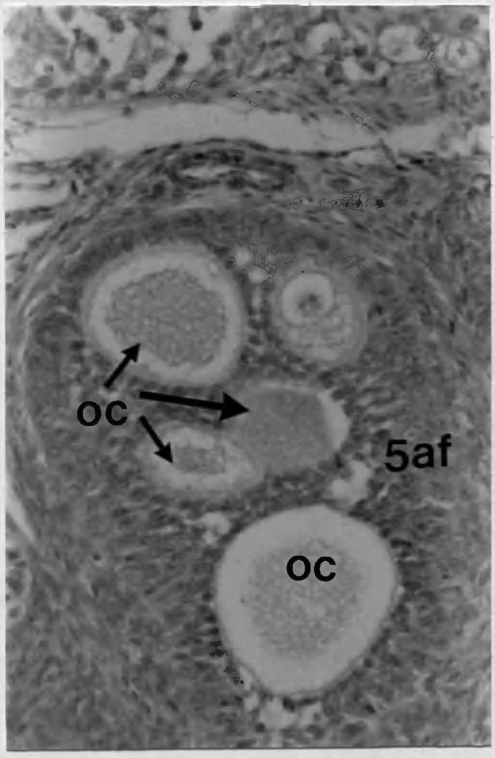


Fig. 3.50 e

Fig. 3.51a. Transverse section through the ovary of a 120 day old pouch young potoroo. a) Note the rete (r), localized in the anterior region of the ovary and the different types of follicles. (Bar = 500 μm).

Fig. 3.51b. Detail of the cortex, showing type 2 follicles (2f) dominating. (Bar = 50 μm).

Fig. 3.51c. Detail of the medulla showing rete tubules (rt) close to a medullary cord (mc). (Bar = 50 μm).

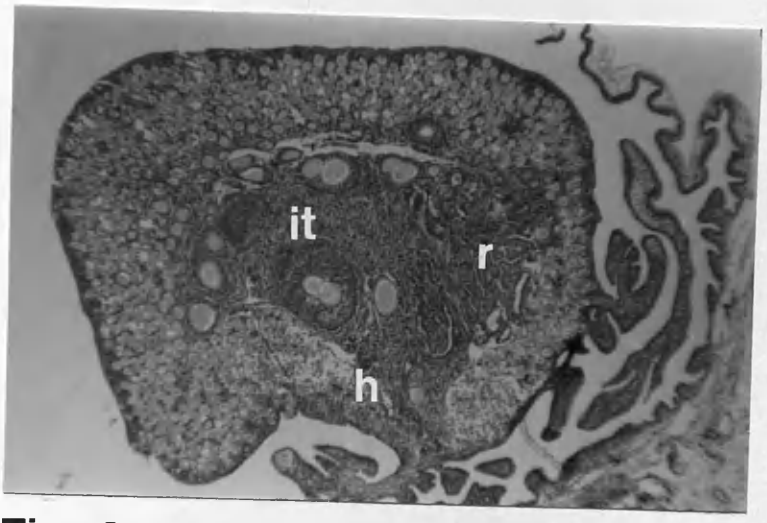


Fig. 3.51a

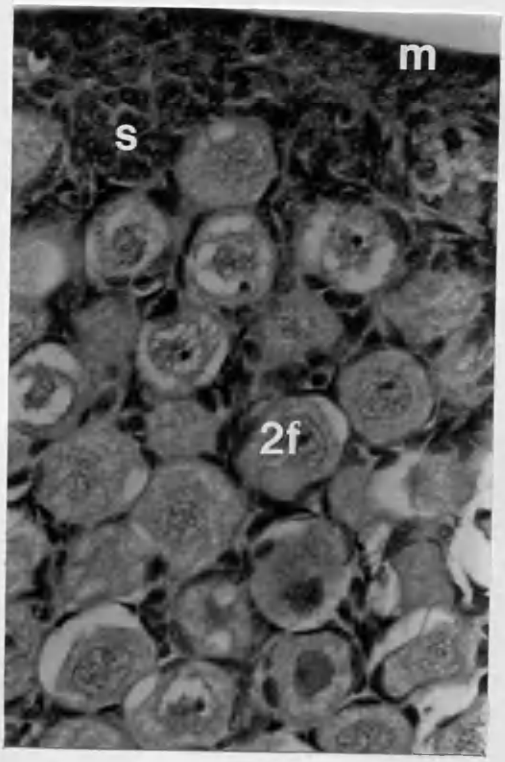


Fig. 3.51b

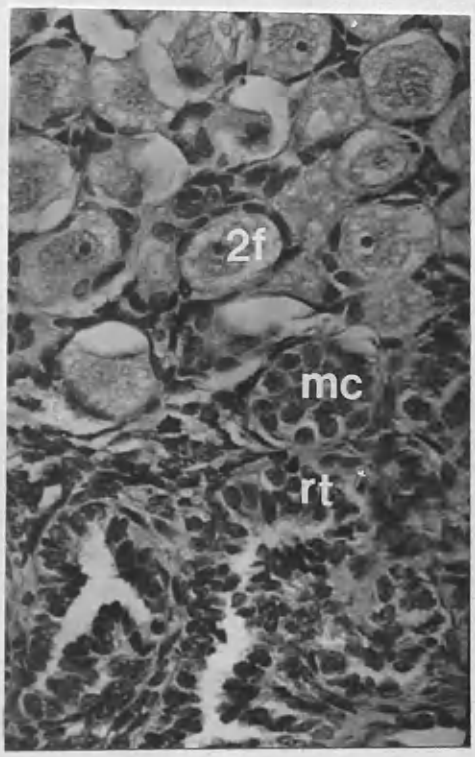


Fig. 3.51c

Fig. 3.51d. Note the degenerating germ cell nests (gcn) in the hilar region, the different types of follicles, and a triovular type 4 follicle (4f). (Bar = 100 μm).

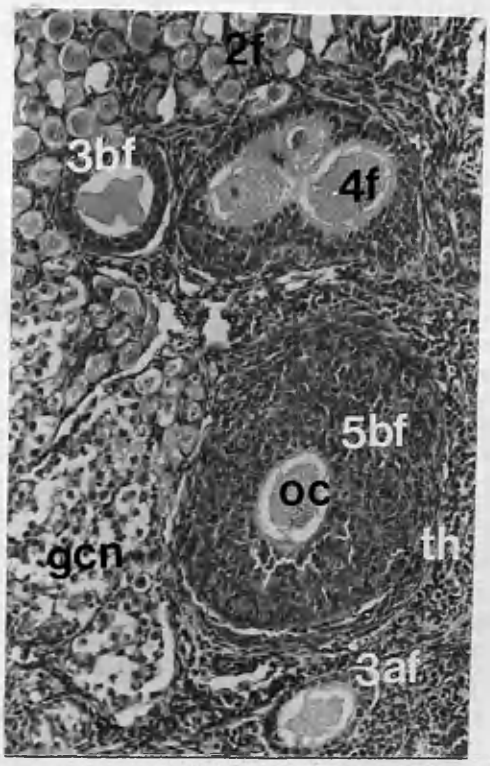


Fig. 3·51d

Fig. 3.52. Transverse section through the ovary of a 2 year old adult potoroo. Note the mesothelium (m), the tunica albuginea (ta) and the medullary-cord type interstitial tissue (it). (Bar = 50 μm).

Fig. 3.53. Transverse section through the ovary of a 3.5 year old adult potoroo. Note different types of follicles and corpora lutea (cl). (Bar = 500 μm).

Fig. 3.54. Transverse section through the ovary of a lactating adult (2.5 years old) potoroo. Note the vascularization, the corpus luteum (cl) and the interstitial tissue (it). (Bar = 500 μm).

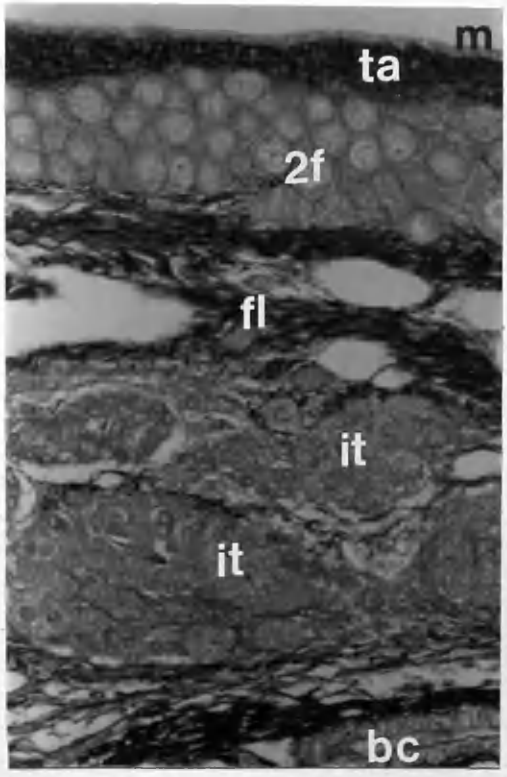


Fig. 3·52

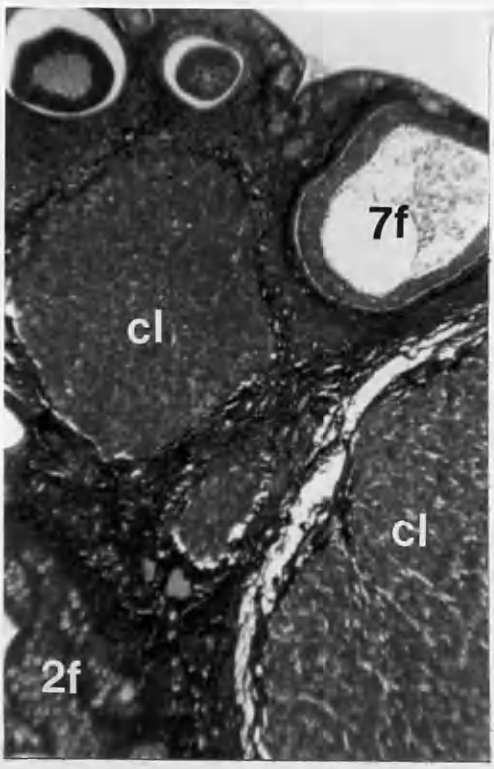


Fig. 3·53

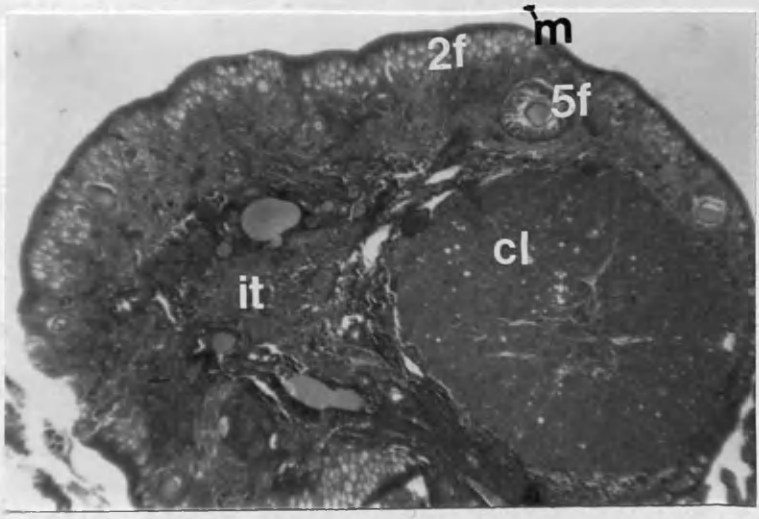


Fig. 3.54

3.4. DISCUSSION

3.4.1. Gonadal Sexual Differentiation

In this study gonad development has been examined in three macropod marsupials namely: the tammar wallaby, Macropus eugenii, the bettong, Bettongia gaimardi and the potoroo, Potorous tridactylus.

In spite of the excellent work of Alcorn (1975) on gonad development in the tammar wallaby there are a few stages which have not been investigated by this author due to their unavailability. Alcorn (1975) studied gonad formation in 21-24 days old embryos; neonate, 12 hour, 3, 5 and 10 days old female pouch young and neonate, 12 hour, 3, 7 and 8 days old male pouch young. The present observations are based on a reinvestigation of the above stages as well as those stages which have not been studied by Alcorn for instance, 2, 4, 6, 8 and 9 days old female pouch young and 4, 5 and 6 days old males.

As reviewed in Section 1.2.2, there is no unanimity in the literature as to the origin of the blastema cells in mammalian gonads. Some authors derive them from the mesothelium of the gonadal ridges or from the mesonephroi; while a dual origin of the blastema cells, from both the mesothelium and the mesonephroi is postulated by others (see p. 9).

In the marsupials (brush-tailed possum, opossum, bandicoot and native cat) investigated by Fraser (1919), the blastema was found to arise from the nephrostomial canals in the embryo. In the tammar wallaby (Alcorn, 1975), bandicoot (Ullmann, 1981a) and native cat (Ullmann, 1984b), the blastema has been derived from the mesonephric mesenchymal cells. Alcorn (1975) states that on day 21 of embryonic life, mesenchymal cells from the ventral margin of the mesonephroi, close to the glomeruli and just adjacent to the lateral margin of the

gonadal ridges, migrate to the latter. In the bandicoot, Ullmann (1981a) reports that at early stages of gonadal development, the irregular columnar mesothelium lacks a basement membrane. Later on, the mesothelium become disorganized and the cells derived from it cannot be distinguished from the blastema.

The observations presented here suggest that in the tammar wallaby both the mesothelial and the mesenchymal cells contribute cells to the blastema. Thus the results are in agreement with those authors who hold to the dual origin of the blastema (Gruenwald, 1942; Torry, 1945; Witschi, 1951; Merchant, 1975, 1979; Pelliniemi, 1975, 1979; Byskov, 1982; Wartenberg, 1983). The results are also comparable to those reported in this work for the mouse (Chapter 2). The contribution of the mesonephroi, however, is different in the two species. While the mesonephric tubules in the mouse give rise to the blastema, in the tammar the blastema derives from mesenchymal cells, located in the ventral margins of the mesonephroi.

The observations presented in this study indicate that on day 24 of embryonic life of the tammar, the mesothelium was 1-2 cells thick, consisting of flattened to cuboidal cells with no basement membrane and no line of demarcation between its cells and the underlying tissue. Some of the mesothelial cells exhibit mitotic activity and the spindles of these cells are perpendicular to the gonadal surface. At later stages of development, that is in 25 day old embryos (a stage just before birth) and up to 2 days post partum, the mesothelium becomes several layers thick and continues to be irregular with many mitotic figures present among its cells. On day 3 of pouch life, the proliferation of the mesothelium in the male gonads ceases and it becomes regular in thickness while the mesothelium continues its proliferation in the ovaries.

The mesonephric tubules of the tammar are well developed and do

not appear to contribute cells to the gonadal blastema, thus confirming Alcorn's (1975) observations. The results presented here also agree with those of Alcorn in the mesonephric mesenchymal origin of the blastema. However the observations indicate that, contrary to Alcorn's (1975) view, the mesothelium also contributes to the blastema. However, he states that the first indication of gonadal ridge formation in the tammar is an increase in height of the more or less cuboidal cells of the mesothelium.

The origin of the blastema in the tammar is different from that reported for the sheep (Zamboni et al., 1979) although both species are similar in having well developed mesonephroi at early stages of gonad development. In the sheep, the caudal half of each mesonephros consists of nephrons while the cranial half is entirely occupied by a single nephron and numerous tubules. The giant nephron is located dorsal to the gonadal ridge. As described in Section 1.2.2, Zamboni et al. (1979) propose that this giant nephron participates in the development of the blastema in this species.

Several views have been expressed in the literature regarding the process of gonadal sexual differentiation (see Section 1.3). These may be briefly summarised as follows:

1) mesothelial proliferation forming the sex cords (Brambell, 1927, see p. 15);
2) cortico-medullary antagonism (Witschi, 1931, see p. 15);
3) induction by mesonephric structures (Merchant-Larios, 1979; Upadhayay et al., 1979; Zamboni et al., 1979; Byskov, 1986, see p. 15);
4) mesonephric-mesothelial cellular interaction (Wartenberg, 1981, 1983, see p. 15);
5) "autodifferentiation" of the blastema cells of the indifferent

gonad (Jost, 1973; Ullmann, 1981a, 1984b, see p. 16).

Sexual differentiation in marsupials has been studied in the bandicoot (Ullmann, 1981a), native cat (Ullmann, 1984b) and tammar wallaby (Alcorn, 1975; Shaw et al., 1987). This study reports on sexual differentiation in the tammar wallaby, bettong and the potoroo.

In the bandicoot, the primary sex cords in the testis differentiate randomly and are secondarily displaced to a peripheral position where they form a coherent zone just below the tunica albuginea (Ullmann, 1981a). Testis development in the native cat Dasyurus viverrinus is similar to that reported for the bandicoot (Ullmann, 1984b). This author states that in neither case do the sex cords have a mesothelial origin. In both the bandicoot and the native cat the sex cords form by the aggregation of the pre-Sertoli cells around the peripherally located PGCs (Ullmann, 1984b). Similar observations have also been reported for some eutherians, for instance cattle: Jost et al., 1975; rhesus monkey: Dong & Fuquet, 1979; human and pig: Pelliniemi et al., 1979 (quoted from Ullmann, 1984b).

As is reported by Alcorn (1975) and in this study, gonadal sexual differentiation in the male tammar wallaby initiates at day 3 of pouch life. More recently, Shaw and his co-workers (1987) found that some aspects of somatic sexual dimorphism occur prior to gonadal sexual differentiation in this species. These investigators observed that at birth, the time when gonadal sexual differentiation has not yet commenced, the scrotal anlagen and the mammary glands are already formed in the genetic male and female pouch young respectively. They conclude from their observations that the appearance of somatic sexual dimorphism before gonadal sexual differentiation is due to an extra gonadal effect of the sex chromosomes rather than to the gonadal hormones since they observed no sex differences in the volumes of the

Mullerian and Wolffian ducts or in the genetically male and female gonads.

In the potoroo and the bettong studied here, sexual differentiation has already occurred by birth since the testes can be distinguished by the presence of the tunica albuginea. Due to the difficulties in getting hold of embryonic stages of these two species, this study has been unable to establish whether somatic sexual dimorphism precedes gonadal sexual differentiation in these two rat kangaroos as it does in the closely related tammar wallaby.

The results presented in this study regarding gonadal sexual differentiation in the tammar, the bettong and the potoroo are in agreement with those for the rat (Jost, 1973), the bandicoot (Ullmann, 1981a) and the native cat (Ullmann, 1984b). It was found that on day 24 of fetal life in the tammar and day 1 of pouch life in the bettong and the potoroo the blastema differentiates into two cell types: a few small fibroblast-like cells and larger irregular cells which form the bulk of the blastema. On day 3 in the tammar and day 1 in the bettong and the potoroo post partum, some of the small fibroblast-like cells are found at the periphery of the putative testes just below the mesothelium and form the tunica albuginea. On day 3 in the tammar and day 2 in the bettong the larger blastema cells, at this stage clumped together, exhibit low affinity for the stains used and enclose the germ cells forming the seminiferous cords. In the tammar on days 4 and 5 the cords become well developed and consist of dividing prespermatogonia and pre-Sertoli cells. The tunica albuginea becomes several layers thick.

Alcorn (1975) indicates that due to the unavailability of 4-6 day old tammar pouch young the exact time for the development of seminiferous tubules is not known but that they are well developed by day 7 of pouch life. The results presented here show that the

seminiferous tubules first appear on day 6 of pouch life.

The observations reported in this work confirm Alcorn's results in that in the tammar the seminiferous tubules appear to radiate from the hilar region. This arrangement of the sex cords is totally lacking in the bandicoot and native cat (Ullmann, 1984b). Instead, the centre of the gonad is initially devoid of sex cords and is exclusively occupied by stromal cells. The cords appear to loop peripherally within the testes and then converge on the rete in the hilar region. The arrangement of the seminiferous cords in the tammar thus differs from that reported for other marsupials, namely the bandicoot and the native cat (Ullmann, 1984b).

The first indication of testicular differentiation in the bandicoot is the formation of the incipient tunica albuginea followed by the transformation of the blastema cells into the pre-Sertoli cells and stroma cells (Ullmann, 1981a). Alcorn (1975) reports the reverse of this in the tammar. In the native cat, however, Ullmann (1984b) shows that both the tunica albuginea and the seminiferous cords appear simultaneously.

The results presented here for the tammar wallaby are in agreement with those reported for the native cat. Both the tunica albuginea and seminiferous cord development were detected on day 3 of pouch life. On the other hand, the observations reported in this study indicate that in the potoroo and the bettong, the first sign of testicular differentiation is the development of the tunica albuginea. The results are thus consistent with those reported for another species of marsupial, namely the bandicoot (Ullmann, 1981a).

3.4.2. Ovary development

The general pattern of ovary development in the macropods studied, that is in the potoroo and the bettong, is similar to that

reported for eutherians (mouse: Peters & Pedersen, 1967; Odor & Blandau, 1969; hamster: Challoner, 1975; rabbit: Peters et al., 1965; Deanesly, 1975; ferret: Deanesly, 1970; pig: Deanesly, 1975; cattle: Ohno & Smith, 1964; human: Pelliniemi, 1979; Baker & Scrimgeour, 1980; Motta & Makabe, 1982) and for marsupials (tammar wallaby: Alcorn, 1975). There is similar oogonial proliferation, formation of germ cell nests, onset of the prophase of the first meiotic division, development of tunica albuginea ovarii, oocyte degeneration, development of different kinds of follicles and atresia of juvenile follicles.

In contrast to most eutherian mammals, where ovarian development occurs prenatally, ovarian growth and differentiation in marsupials occur postnatally during the pouch life of the young. Postnatal oogenesis, however, has been described for a few mammalian species, for instance the rabbit: Peters et al., 1965; Gondos, 1969; Deanesly, 1975; hamster: Challoner, 1974 & 1975; Deanesly, 1974 & 1975 and the ferret: Deanesly, 1970).

As is mentioned in Section 1.3.1, two kinds of ovarian development have been described in mammals. The first category of mammalian species is characterized by immediate meiosis: the oogonia enter meiosis simultaneously or shortly after the onset of gonadal sexual differentiation as in the case of the mouse, rat and man (Byskov, 1986). On the other hand, a lag period separates gonadal sexual differentiation from the onset of meiosis in those species with delayed meiosis, as in the case of the pig, rabbit, cow and sheep (Byskov, 1986). In these species, the oogonia become enclosed in cell nests that resemble testicular cords (see Section 1.3).

Bettong and potoroo ovaries are characterized by immediate meiosis. At the time when the testes differentiate (on post partum

days 2-3 in the potoroo and day 7 in the bettong) the ovaries are compact structures consisting of uniformly distributed germinal and blastemal cells as described for the ovaries of those species with immediate meiosis. The germ cell cords which occur in the ovaries of those species with delayed meiosis, were detected neither in the potoroo nor in the bettong. On day 15 of pouch life, the blastema differentiates into two cell types: small fibroblast-like cells and larger irregular cells. The proliferating female germ cells become peripherally located forming the cortex. In the potoroo the oocytes were detected at the prophase of the first meiotic division around day 28 of pouch life and in the bettong they were first observed in meiosis on day 42. The difference in the time of initial detection of meiosis may be due to the fact that comparable stages in ovary development were missing in the bettong. A thin layer of connective tissue separates the cortex from the medulla around days 15 and 16 in the potoroo and the bettong respectively. By day 28 (in the potoroo) and 42 (in the bettong), the tunica albuginea is present.

Different views are expressed in the literature regarding the development of the rete, which has been derived from the mesothelium (Allen, 1904; Felix, 1912; Morgan, 1943), the mesonephroi (Byskov & Lintern-Moore, 1973; Stein & Anderson, 1979) or from cells present in the anterior of gonadal ridges (Fraser, 1919; Alcorn, 1975; Deanesly, 1975; Ullmann, 1984b).

Byskov and Lintern-Moore (1975) studied the development of the rete in the mouse from the day of birth until day 14 post partum. They recognize 3 regions of the rete which are: the "extraovarian rete" which is found in the periovarian tissue, the "intraovarian rete" which is observed within the ovary and the "connecting rete" which joins the extra and the intra ovarian rete. These workers observed that at birth the extraovarian rete ends blindly in a single wide tube

in the periovarian tissue and the connection with the Wolffian duct disappears. Upon reaching the ovary it branches forming compact cell cords, the connecting rete. The latter branches again into tiny cell cords or tubes, forming the intraovarian rete; this in turn branches and occupies the major part of the medulla and extends into the cortex where it "widens" to enclose both single oocytes and nests of oocytes.

The blastema has also been considered as a source of granulosa cells (Peters & Pedersen, 1967; Odor & Blandau, 1969; Peters et al., 1969; Deanesly, 1970; Alcorn, 1975; Ullmann, 1984a). Byskov (1975) working on the cat, mink and ferret derives the granulosa from both the mesothelium and the mesonephroi. Byskov (1978) reports the presence of membrane-bound bodies (dense rete bodies) in the cytoplasmic extensions of the migrating mesonephric cells (according to her, the rete cells) in mouse ovaries. This cytoplasmic marker is said to be absent from other somatic cells and thus appears to be specific to the rete. Byskov (1986) finds that these dense rete bodies are neither microperoxisomes nor lysosomes, since they showed negative reactions with peroxidase and acid phosphatase. She states that the function of the dense rete bodies is still unknown and that similar inclusions are also found in the germ cells. It is suggested that in both cases these bodies are related to interactions with surrounding cells.

Alcorn (1975) working on the tammar wallaby describes the presence of vesicles in the cytoplasm of the blastema cells and the rete cells. This study (p. 83) shows that membrane bound dense bodies also occur in the blastema cells of newborn potoroo ovaries. These observations and those reported by Alcorn (1975) indicate that these inclusions are not exclusively confined to the rete cells, as is suggested by Byskov (1978), but that they are present also in other

somatic cell types.

Rete development has been described in a number of marsupials - the brush-tailed possum, bandicoot, American possum and the native cat - by Fraser (1919). This author states "... the rete ridge is simply the reduced anterior continuation of the genital ridge". More recently, Ullmann (1984b) reinvestigated her slides of bandicoot embryos and confirmed Fraser's observation regarding the development of the rete. Ullmann (1984b) states that the rete forms as a result of cell condensations (presumably mesenchymal cells) at the anterior end of the gonadal ridges. Alcorn (1975) also agrees with Fraser (1919) and believes that rete development in the tammar wallaby is identical to that described for the brush-tailed possum.

The development of the rete in the potoroo is similar to that reported for other marsupials. The rete forms as a result of a cell condensation at the anterior region of the gonad, forming a cord which later on branches in the gonad.

While in the brush-tailed possum (Ullmann, 1986) and the potoroo studied here the rete branches deeply in the ovaries, it was found that in the bandicoot it ends blindly in the hilar region (Ullmann, 1984a).

Several views are expressed in the literature regarding the origin of the granulosa (follicle) cells and these have been reviewed in Section 1.3.1.2. Some authors derive them from the mesothelium of the gonadal ridges (Brambell, 1927; Everett, 1943; Franchi et al., 1962; Gondos, 1969; Motta & Makabe, 1982), others from the mesonephroi: either from the mesonephric tubules (Upadhayay et al., 1979; Zamboni et al., 1979) or from the rete (Byskov & Lintern-Moore, 1973; Stein & Anderson, 1979).

In marsupials only two studies are available describing the origin of the granulosa cells. Alcorn (1975) working on the tammar wallaby

and Ullmann (1984a) working on the bandicoot derive the granulosa from the blastema. The former found that the granulosa cells arise from the proliferation of mesenchymal cells of the septae between the germ cell nests and from those migrating into the cortex from the medullary stroma. Alcorn states "In both cases, the mesenchymal cells originated from the cells of the central somatic blastema". Ullmann (1984a) believes that the blastema, at early stages of ovary development, differentiates into two cell types: small fibroblast-like cells and larger cells. The latter clump together forming medullary cords which grow through the fibrous layer which separates the cortex from the medulla and surround the oocytes to become granulosa cells. She found that in bandicoot ovaries the medullary cords were large and distinct.

The observations reported here regarding the origin of the granulosa cells in the potoroo and the bettong are in agreement with those reported for some eutherians (mouse: Peters & Pedersen, 1967; Peter et al., 1969; ferret: Deanesly, 1970) and the two marsupials already investigated (Alcorn, 1975; Ullmann, 1984a). The way in which the blastema contributes to the granulosa is similar to that described for the bandicoot and brush-tailed possum (Ullmann, 1984a).

During the first week of pouch life (day 2-3 in potoroo and day 7 in bettong), the blastema differentiates into 2 cell types: small fibroblast-like cells with dense nuclei and larger cells with irregular nuclei. In the ovaries of 15 day old pouch young of the potoroo and the bettong, the larger cells clump together in the centre forming medullary cords. The latter grow and penetrate the fibrous layer which separates the cortex from the medulla. Eventually the cords enclose the oocytes which occupy the innermost zone of the cortex.

No evidence was obtained for the derivation of the granulosa from the rete. It was found that at the time when the medullary cords were developed the rete was present at the hilar region but did not yet penetrate the ovary.

At later stages of development, in both species, the rete penetrates the anterior end of the ovary and makes contact with the medullary cords locally. Although the rete penetrates the ovary of the potoroo very deeply, it was observed that the oocytes at a distance from the rete were already surrounded by granulosa cells. That is to say, folliculogenesis is in progress at one end while the rete is localised at the other end of the ovary. Thus it is unlikely that the rete gives rise to granulosa cells in these marsupials as suggested by Byskov and Lintern-Moore (1975) for the mouse. The particular non-involvement of the rete in the development of the granulosa is even more clearly illustrated in the bandicoot (Ullmann, 1984) where the rete ends blindly at the hilar region and is histologically quite different from the medullary cords which are more distinct than in the macropods. However, it is possible that the structures here identified as medullary cords have been interpreted as the intraovarian rete by Byskov and Lintern-Moore (1973).

It has been suggested that the rete triggers the onset of meiosis in female germ cells (Baker & Neal, 1973; Byskov, 1974). The former investigators state that it is not known whether the action of the rete depends on direct cellular contact between the rete and the germ cells or on a diffusible substance. O' and Baker (1976) report that in the hamster meiosis initiates on day 15 of gestation. When they cultured fetal ovaries with or without the rete, on day 12 and 13 post coitum, they observed that in ovaries cultured with the rete for 14 days most of the oogonia reached the leptotene stage of meiotic prophase. On the other hand, apart from a few oocytes found at the

leptotene stage the majority of oogonia did not enter meiosis in ovaries cultured for 4-6 days without the rete. O' and Baker (1976) found that the oogonia and the oocytes became atretic after 8 days in culture without the rete and by day 12 the ovaries were devoid of germ cells. These investigators do not refer to follicle formation. It is, therefore, important to ascertain if folliculogenesis initiates in ovaries cultured without the rete.

A few oocytes may occasionally be found within the rete and the medullary cords in the hilar region and in the medulla. Similar observations have been reported also in the ferret, rabbit and pig (Deanesly, 1975) and are described by the latter as follows: "... active differentiation and proliferation of the rete coincided with the later stages of the meiotic prophase and the degeneration of many oocytes both in the cortex and the medulla. During early development in the ferret, numerous oogonia were carried into the ovarian medulla and gave rise to oocytes which passed into various meiotic stages and were "taken up by rete tubules", as were some of the oocytes degenerating in the cortex. It is not clear from Deanesly's account how the oocytes enter the tubules: whether it is through migration, by phagocytotic activity of the cells which constitute the tubules or by other unknown means of movement. The first possibility can be excluded since it has been found that the germ cells lose their migratory behaviour once they become settled in the gonads (Byskov, 1986). Secondly, since the rete tubules are surrounded by a basement membrane, it is difficult to visualise them undergoing phagocytotic activity. Deanesly (1975) found that the oocytes which are "trapped" in the rete were smaller than the healthy oocytes in primordial follicles and she considers them as "surplus oocytes".

In the tammar wallaby, Alcorn (1975) reports the presence of

medullary cords but does not believe in their involvement in granulosa development. He states that on day 8 of pouch life, the central somatic blastema differentiates giving rise to medullary cords in the centre of the ovaries and the rete cords in the hilar region. He claims that since both these structures have the same origin they should have a common name, the rete cords. According to him the intra, the connecting and the extraovarian rete of Byskov and Lintern-Moore (1975) are equivalent to the medullary and hilar cords, the ovarian efferent ducts and the epoophoron respectively.

On day 12 of pouch life in the tammar, Alcorn distinguishes 2 types of cells in the rete cords: types A and B. The former are subspherical with indistinct cell membranes while type B cells are elongate and have distinct cell membranes.

Although type B occur in large numbers (80-90%) at this stage, he observed that the rete cords of 95 day pouch young ovaries were dominated by type A cells. At the ultra-structural level, however, Alcorn describes only one type of rete cell (and does not make reference to cell types A and B) which is similar to the blastema cells of the newborn's ovary in many respects.

Alcorn's observations of 2 cell types in the medullary cords could not be confirmed for the tammar, bettong or potoroo.

In this study, the term medullary cords is used for the cell cords which are found in the medullary region while the term rete has been used to describe the cords and tubules which are found at the hilar region at early stages of development and branch in the ovaries at later stages. Medullary cords were first observed on day 32 of pouch life in potoroo.

There is no unanimity in the literature as to the origin of the interstitial tissue (IT). Some authors derive it from the stroma (Allen, 1904; O'Donoghue, 1916; Gruenwald, 1942; Deanesly, 1970;

Quattropani, 1973; Pelliniemi, 1975b; Pehleman & Lombard, 1978), others maintain that the theca interna of atretic follicles gives rise to IT (Kingsbury, 1939; Dawson & McCabe, 1951; Mori & Matsumoto, 1970; Mossman & Duke, 1973). Allen (1904) claims that the IT in the pig and rabbit derives from the theca interna which has a stromal origin.

In the ferret Deanesly (1970) observed that from day 7 a few lipid containing cells (the precursors of the IT) appear in the ovaries, originating in the medullary stroma. They vary in size and shape and they eventually increase in size and differentiate into epitheloid lipid containing cells with rounded nuclei. Deanesly (1970) reports that these cells, which form granular lobules, were first detected at the hilar region and later on occupy most of the medulla. She found that IT gave a positive reaction when stained for lipids while the cells of the rete tubules did not do so, indicating that they lacked stored lipids.

Quatropani (1973) and Pehleman and Lombard (1978), working on mouse ovaries, observed that the IT became fully differentiated and had the characteristic features of steroid secreting cells at day 9-10 post partum. The cells had spherical nuclei, large numbers of lipid droplets, well developed ER and spherical mitochondria with tubular cristae. Quatropani (1973) states that the lipid droplets have electron-lucent cores and an opaque periphery. This author noted that the first appearance of the IT differed in different strains of mice. In C57BL/6RQ mice IT first become detectable on day 10 post partum; in C57BL/6J strain of mice on day 12 while in yet another strain of mice it was first seen on day 9 post partum (Pehleman & Lombard, 1978).

In the tammar wallaby, Alcorn (1975) derives the IT from the medullary and hilar rete cords. Since Alcorn derives these cords from the blastema, the IT has a blastemal origin too.

The observations on the origin of the IT in the potoroo are similar to those in the tammar in as much as it is derived from the blastema. In the potoroo the IT derives from the medullary cords which have a blastema origin.

However, the IT in the tammar wallaby derives from both the medullary and hilar rete cords, while in the potoroo it was found that the medullary cords were the only source for the IT. No evidence was obtained in this study for the contribution of the rete to the formation of IT. It was found that before the development of IT both the medullary cords and the rete were present in the ovaries and could easily be distinguished from each other (see p. 91). At the time when IT could be distinguished, the rete cords were also present in the ovaries. At later stages of development, when only remnants of medullary cords were present, the rete was still found ramifying at the anterior end of the ovary (see p. 91 & 92). In the potoroo the IT is first recognizable during day 91-92 of pouch life, when the medullary cords become glandular in appearance. The medullary cord cells which have spherical nuclei become hypertrophied. They are found in clusters of various sizes separated from each other by a thin layer of collagen fibres. However, the presence of a transitory stage in the development of the IT was already evident at day 86 (equivalent to developmental age 66-69, see p. 89) of pouch life. The ultrastructural studies of the one potoroo specimen examined at this stage show that the medullary cords are beginning to clump together, lose their basal lamina at the points of contact and possess lipid droplets. The medullary cord cells, however, are not highly differentiated and lack the characteristic features of mature steroid secreting cells. Electron micrographs show the presence of only little smooth ER and a few vesicular or elongated mitochondria having irregularly arranged cristae in the cytoplasm of the IT.

The histochemical tests performed on the ovary of the pouch young at this stage confirm the contention that the medullary cord cells are not well differentiated. No 5 3B HSD enzyme activity was detected in the ovary on day 86 (equivalent to developmental age 66-69 days, see p. 178) of pouch life. Unfortunately, in the absence of ultrastructural and histochemical work on older stages, this study is unable to determine the exact time for the establishment of steroidogenesis during development and this remains to be elucidated.

As is reviewed in Section 1.3.1.4., agreement is lacking as to the origin of polyovular follicles (POFs) which only occur in some mammalian species.

The prevalence of the occurrence in the potoroo seems to indicate that the phenomenon of polyovuly is natural in this species. POFs are found at different stages of development on day 75 of pouch life when follicles of type 3a are polyovular. At this stage a few monovular and biovular follicles are also binucleate. The presence of a high incidence of binucleate ova at early stages of development and at the time when polyovular follicles start to develop suggests that POFs may form as a result of a failure of oogonia to separate from each other and thus become surrounded by a common envelope of follicle cells. The presence of follicles having oocytes of the same size, some of which are in close contact with each other supports this suggestion. However, oocytes with differently sized ova, which are separated from each other by granulosa cells, have also been observed to constitute the POFs. The presence of variably sized ova in a follicle may be due to the differential growth of some of these oocytes or it may be due to a difference in their topographic situation (Al-Mufti et al., 1987, personal communication). It could be that the oocyte has to occupy a certain position within the follicle to be in a suitable environment

for its growth.

The presence of intercellular bridges between oogonia and between oocytes has been described in the ovaries of several mammalian species and related to the synchrony in the division of these female germ cells (rat: Franchi & Mandl, 1962; hamster: Weakley, 1967; rabbit: Zamboni & Gondos, 1968; Gondos & Zamboni, 1969).

This study was unable to provide ultrastructural evidence for intercellular bridges which may exist between the oocytes in potoroo POFs. The presence of oocytes at various stages of meiosis within the same or in adjacent germ cell nests suggests that such bridges may not exist, allowing asynchronous divisions to take place.

The absence of intercellular bridges from the oocytes of biovular follicles was reported by Papadaki (1978) working on an adult human ovary from a patient with amenorrhea who was treated with gonadotropin .

Deanesly (1970) working on ferret, Ioannou (1964) on guinea pig and Baker (1965) and Baker and Franchi (1967) on human ovaries have reported the absence of synchronised divisions among the oocytes in germ cell nests. They found that most meiotic stages could be present within the same nest. Deanesly (1970) states that the degenerative process in the ferret is gradual and is not synchronized; zygotene, pachytene and diplotene stages are all present and it appears that oocytes can finally degenerate at any stage of meiosis. Baker and Neal (1973) state that asynchrony is the rule in the majority of mammalian species.

The lack of synchronous divisions among the oocyte nests makes it difficult to interpret the formation of POFs as a failure of oogonia, which are joined by intercellular bridges, to separate from each other before they become surrounded by follicle cells. Perhaps the presence

of binucleate monovular and biovular follicles before and at the time of the development of POFs is inadequate support for this contention. Thus the other possibility, that of closely apposed oocytes being surrounded by a common envelope of follicle cells may more likely describe the development of POFs. It is also possible that the number of granulosa cells available is not enough for the large numbers of oocytes developing.

In potoroo pouch young POFs at different stages of development, from primordial to antral, have been observed and these may contain from 2 to 20 oocytes within them. Although the POFs occur very frequently in the ovaries of the juvenile potoroos studied, it was found that these POFs become pyknotic and disappear from the ovaries of adults. At day 91-98 of pouch life, when atresia of juvenile follicles is prominent, a few POFs, at different stages of development, undergo atresia. This observation of atretic POFs is consistent with Hartman's (1926) view that POFs are destined to atresia. However, a POF with 2 oocytes was detected in a female adult ovary. The reason for the frequent occurrence of POFs in the ovaries of juvenile potoroos still remains a puzzle.

The ultrastructure of oogonia in the potoroo ovaries is similar to that reported for eutherians (mouse: Odor & Blandau, 1969; rat: Franchi & Mandl, 1962; pig: Anderson & Beams, 1960; human: Baker & Franchi, 1967). Electron micrographs indicate that the oogonia are spherical in shape and are larger than the surrounding somatic cells. The nuclei contain a well developed nucleolus. The mitochondria are vesicular or elongated with irregularly arranged cristae.

Some authors report that the nuclear envelope of the oogonium has a regular contour (Franchi & Mandl , 1962; Baker & Franchi, 1967) while others find that the nuclear membrane is irregular (Odor & Blandau, 1969). The observations presented in this work are similar to

those of Odor & Blandau (1969). It was found that the nuclei are irregular and may be indented. The heterochromatin is either distributed throughout the nucleus or is restricted to the periphery in a beaded configuration. Similar observations have been made for the heterochromatin in the primordial germ cells (PGCs) in some eutherian and marsupial mammals (mouse: Clark & Eddy, 1975; tammar wallaby: Alcorn, 1975; bandicoot: Ullmann, 1981b).

In contrast to the oogonia of the mouse which only contain sparse ribosomes (Odor & Blandau, 1969), those of the potoroo are characterized by the presence of abundant ribosomes, a feature similar to that reported for the rat (Franchi & Mandl, 1962).

The oogonia of the potoroo are characterized by the presence of a large quantity of rough endoplasmic reticulum which may be associated with the mitochondria, the nuclear envelope or the plasma membrane. These observations contrast with those described for eutherians (Odor & Blandau, 1969; Byskov, 1986). The former report that rat oogonia contain sparse endoplasmic reticulum.

Electron micrographs indicate that in the potoroo the oocyte cytoplasm is less electron dense than in the surrounding follicle cells. This may be due to the growth of the oocytes and the dispersion of the ribosomes, as suggested for the mouse. Similar observation was noted for the oocyte by Odor and Blandau (1969). On the contrary, the number of mitochondria and Golgi complexes have increased and this is in accordance with observations reported for other mammalian species (mouse: Odor & Blandau, 1969; bandicoot: Ullmann, 1978).

The ultrastructural studies did not reveal the presence of ooplasmic localizations such as the paranuclear complex, the veiscle microtubule complex or the aggregate of tubular cisternae, which were found in the primordial oocytes of the bandicoot (Ullmann, 1978). This

author was able to identify the paranuclear complex even at the light microscopic level. She states that "In paraffin sections the paranuclear complex (PNC) appears as an eosinophil body which is usually ovoid in outline and situated close to the nucleus, but variations in its shape, size, number and position are not uncommon."

The observations presented here show that the ovaries of adult potoroos are similar to those described for other mammalian species (Mossman & Duke, 1978). The ovaries are surrounded by a regular mesothelium with a thin basement membrane. The cortex contain sparse follicles. The secondary follicles are enclosed in a theca made up of two layers: the theca interna and the theca externa. The latter may be inconspicuous or absent from the ovaries of many mammalian species (Mossman & Duke, 1973).

Mossman and Duke (1973) state that the corpora lutea eventually degenerate. "In large mammals, for instance in man, fibrous, often pigmented 'scars', corpora albicantia, mark their sites, sometimes for many months, but in most smaller mammals, their degeneration is rapid and no trace remains". This may explain why a corpus albicans was found in only one out of 12 adult ovaries examined in this study.

Table 3.2 was constructed according to developmental criteria mainly from specimens donated by different sources (see p. 74). Generally this coincided with the chronological sequence, but a few exceptions were observed (pt 1, 2, 4, 94) notably specimen pt. 38. This specimen which was bred in Glasgow was at least 86 days old (see Appendix), yet its developmental age was only 66-69 days. The explanation for this discrepancy between developmental and chronological age is at present unknown. Most of the donated specimens used were collected from the wild; the specimens held in Glasgow derived from a stock which has been inbred for about 20 years (Ullmann & Brown, 1983). This fact may account for the noted discrepancy.

3.5. RESULTS AND DISCUSSION OF QUANTITATIVE STUDY

Quantitative data related to the development of the ovaries in the potoroo are recorded in Table 3.3 and Figs. 3.55, 3.56, 3.57.

It was found that the volume of the ovary increases steadily from 0.014409 mm^3 on day 5 *post partum* to 3.733902 mm^3 on day 120 of pouch life.

The increase in the volume of the ovary may be due to growth of the cortical rather than the medullary region: the volume of the cortex increases from 0.3245 mm^3 on day 29 to 2.7657 mm^3 on day 120 of pouch life (Fig. 3.55) while there is only a slight increase in the volume of the medulla (Fig. 3.56). The volume of the latter increases from 0.0455 mm^3 on day 29 to only 0.9682 on day 120.

In adult females (2 years old) however, the volume of the medulla is greater than that of the cortex. It was found that while the volume of the latter reaches 6.8462 mm^3, that of the medulla increases to 8.5573 mm^3. Although there is not much difference between the volumes of 2 and 4 year old adult ovaries, dissimilarity exists between the volume of the cortex and medulla. While the volume of the cortex reaches 9.195 mm^3 in the 4 year old ovary, it increases to only 6.846 mm^3 in the case of the 2 year old animal. Conversely, medullary volume is higher in the 2 year old animal than in the 4 year old one, being 8.557 mm^3 and 6.509 mm^3 respectively. This may be due to physiological reasons. Individual differences may be expected since ovaries may differ in the number of the growing large follicles.

It is reported that in eutherian mammals the ovary shows a steady increase in volume throughout development (rat: Beaumont & Mandl, 1962; guinea pig: Ioannou, 1964; cow: Erickson, 1966; rhesus monkey: Baker, 1966 and human: Baker, 1963). On the other hand Alcorn (1975), estimating the volume of the ovary in the tammar wallaby, states that

ovarian volume does not increase steadily with age and that the ovarian volumetric growth curve can be divided into 4 distinct stages. The first stages - from birth to 22 days - represents a period of steady growth; the second stage from 22 days to 50 days, represents a period of more rapid growth, the third stage - from 50 days to about 110 days - represents a period of little or no growth and the fourth stage - from 110 days onwards is a period of rapid growth.

The results presented in this study for the potoroo are thus consistent with those reported for eutherians and at variance with the situation in the tammar wallaby, its close relative. However, the observations here indicate that there is a slight variation between different specimens of the same age (days 29, 48 and 98 of pouch life, Table 3.3). Similar findings are reported in the rat (Beaumont & Mandl, 1962) and human (Baker, 1963) and it is suggested by the latter author that this may be partly due to the use of two different fixatives or differences in the duration of fixation. The former reason may apply in this study since different fixatives were used.

Table 3.3 Ovarian, cortical and medullary volumes in the potoroo

Age	Volume of ovary (mm^3)	Volume of cortex (mm^3)	Volume of medulla (mm^3)
5 days	0.014409		
9 "	0.029627		
20 "	0.075274		
29 "	0.371012	0.324484	0.046528
29 "	0.457495	0.357052	0.100443
37 "	0.582368	0.512262	0.070106
38 "	0.697456	0.592914	0.104542
40 "	0.751578	0.602737	0.148841
41 "	0.265116	0.207708	0.057508
48 "	1.566244	1.287984	0.278260
48 "	1.992328	1.561118	0.431210
50 "	2.013264	1.881508	0.229756
50 "	1.920009	1.762114	0.157895
70 "	1.550989	1.329712	0.221277
82 "	3.154260	2.678226	0.476034
82 "	3.087941	2.669253	0.418688
91 "	2.514544	1.841196	0.673348
91 "	2.368736	1.833339	0.535397
98 "	3.973572	2.492598	1.480974
98 "	3.355906	2.451929	0.903977
120 "	2.703361	1.920451	0.782910
120 "	3.733908	2.765722	0.968186
2 years	15.403465	6.846162	8.557303
4 "	15.70299	9.194362	6.508628

Fig. 3.55. Mean volume of ovary and cortex during development.

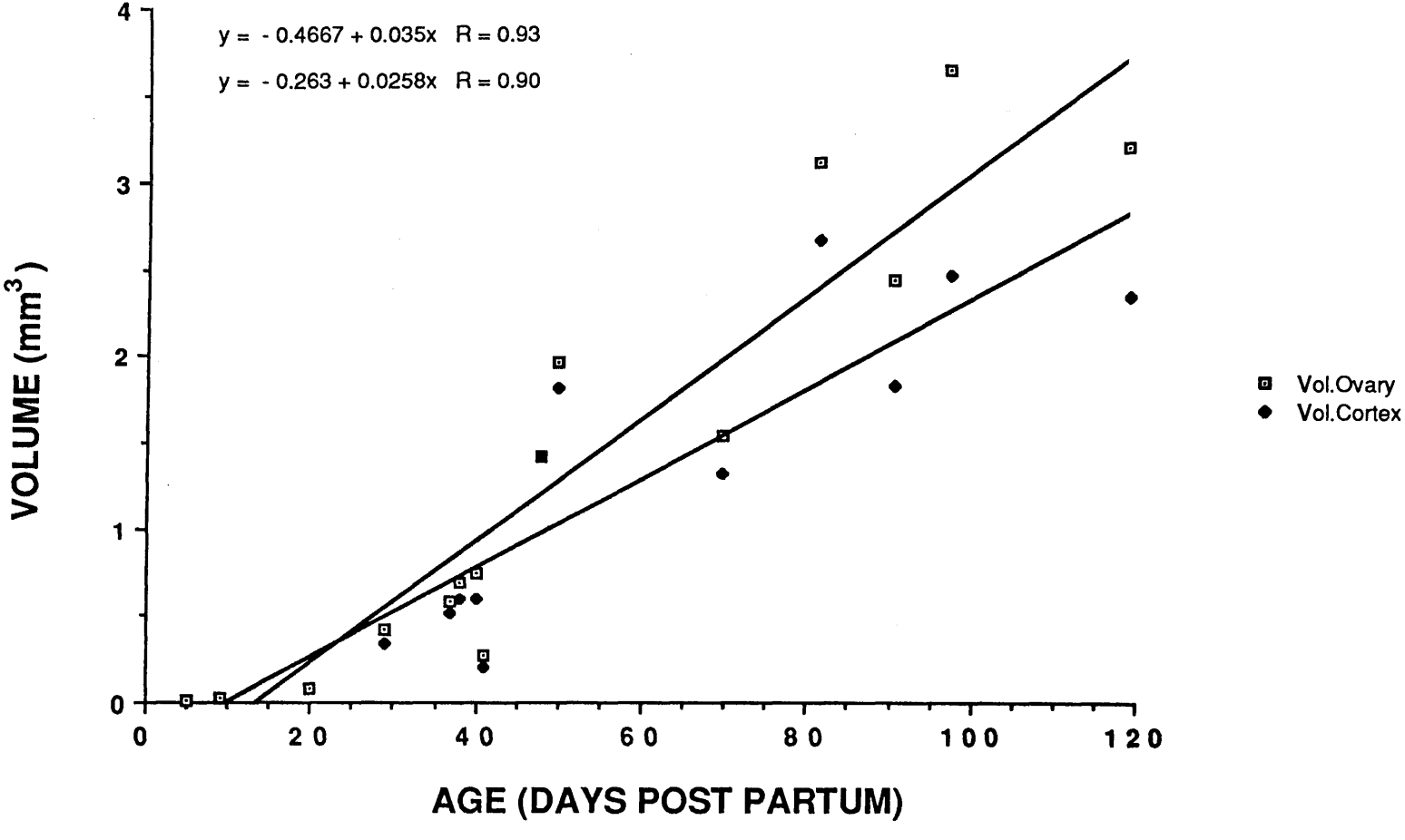

y = - 0.4667 + 0.035x R = 0.93
y = - 0.263 + 0.0258x R = 0.90
VOLUME (mm3)
AGE (DAYS POST PARTUM)
0
1
2
3
4
0
20
40
60
80
100
120
Vol.Ovary
Vol.Cortex

Fig. 3.55

Fig. 3.56. Mean volume of ovary and medulla during development.

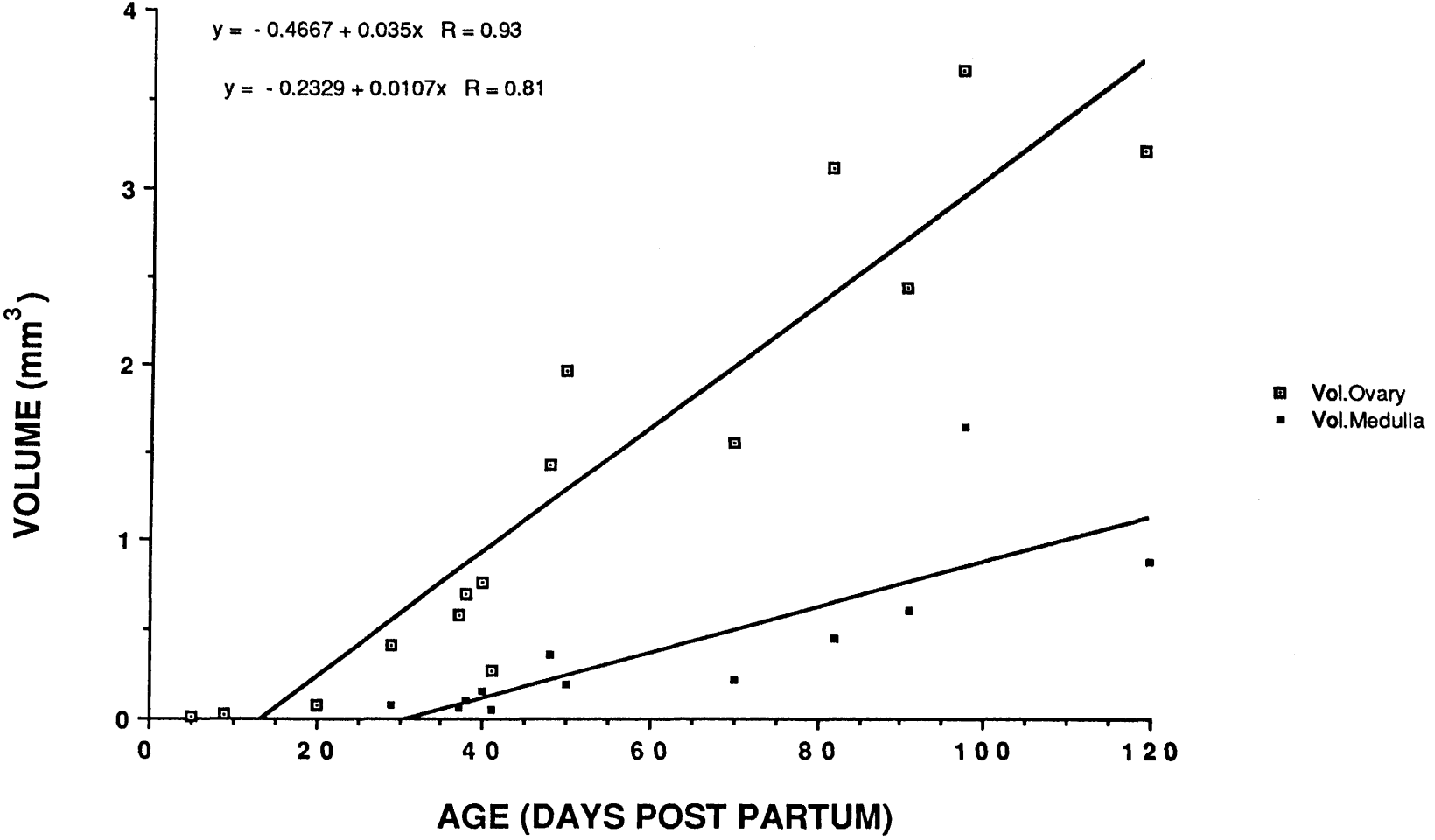

y = - 0.4667 + 0.035x R = 0.93
y = - 0.2329 + 0.0107x R = 0.81
VOLUME (mm3)
AGE (DAYS POST PARTUM)
0
1
2
3
4
0
20
40
60
80
100
120
Vol.Ovary
Vol.Medulla

Fig. 3.56

Fig. 3.57. Mean volume of cortex and medulla during development.

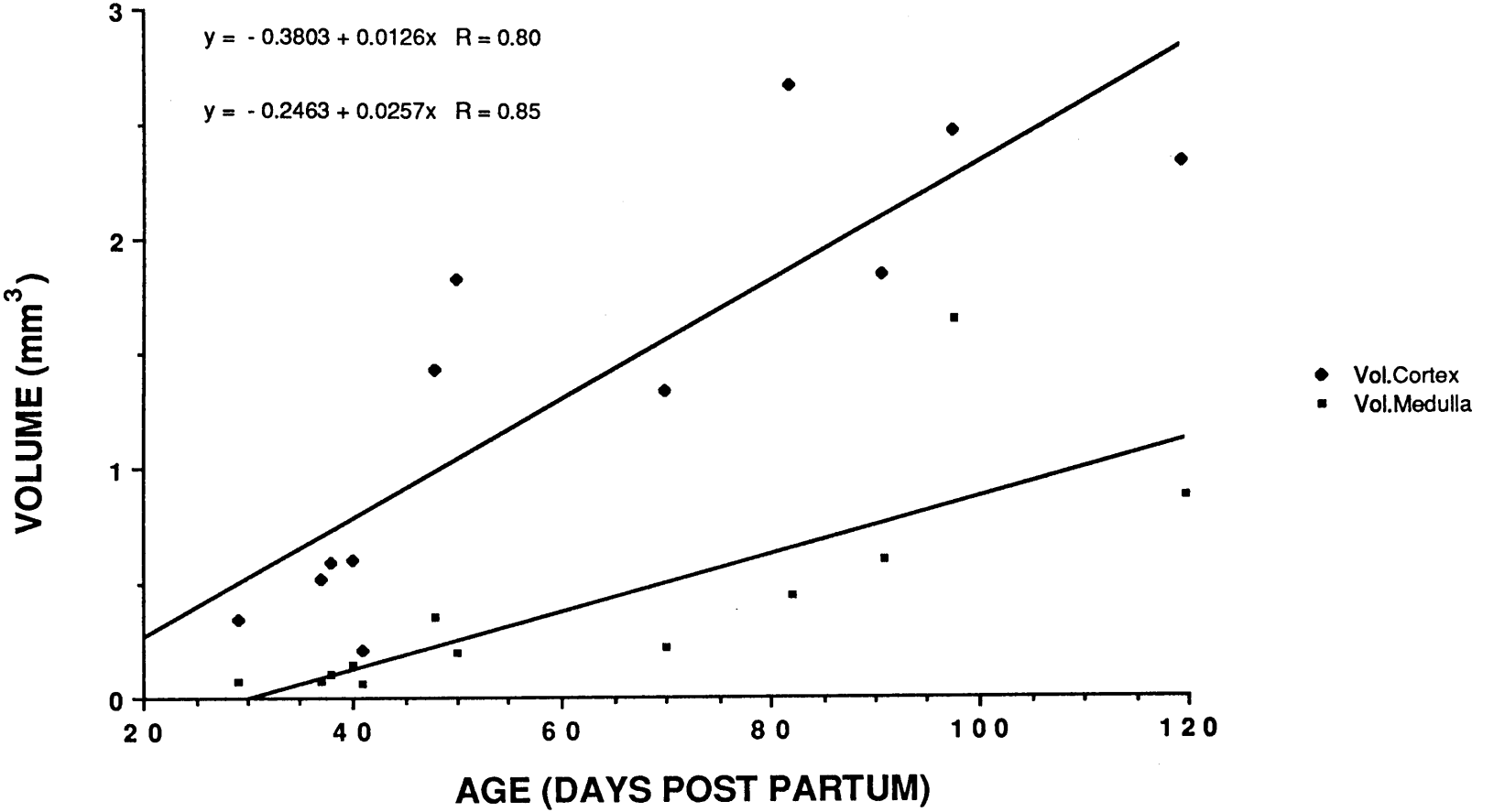

y = - 0.3803 + 0.0126x R = 0.80
y = - 0.2463 + 0.0257x R = 0.85
VOLUME (mm3)
0
1
2
3
20
40
60
80
100
120
AGE (DAYS POST PARTUM)
Vol.Cortex
Vol.Medulla

Fig. 3.57

CHAPTER FOUR

METHODS OF DEMONSTRATION OF HYDROXYSTEROID DEHYDROGENASE ENZYME IN THE OVARIES OF MACROPOD MARSUPIALS

4.1.1. INTRODUCTION

Enzyme histochemical studies have provided the most interesting and important information about the histochemstry of the ovary.

Hydroxysteroid dehydrogenase (5, 3B HSD) is an enzyme directly involved in the biosynthesis of most biologically active ovarian steroid hormones (Wattenberg, 1958; Jacoby, 1962; Motta, 1970; Pehleman & Lombard, 1978).

HSD is essential in the conversion of pregnenolone to progesterone and of dehydroepiandrosterone to androsterone, precursors of androgens, oestrogens and corticosteroids (Bjersing, 1967; Goldman, 1970; Haffen, 1970). HSD acts to remove an atom of hydrogen in position C-3 from the steroid molecule (Haffen, 1970). Thus the presence of such an enzyme in tissue is evidence of steroidogenic capability (Guraya, 1972). HSD was first demonstrated histochemically by Wattenberg (1958).

As mentioned in (1.3), the primordial germ cells (PGCs) migrate from their site of origin, along the hindgut to the presumptive gonads (Wylie, 1986). Chemotaxis has been postulated to guide PGCs from the epiblast to their final destination in the gonadal ridges (Wylie, 1986, see section 1.3). The finding of HSD activity in the gonadal ridges of human fetuses raises the possibility that HSD may be the chemotactic factor (Baillie et al., 1966).

The influence of the fetal testes on the growth and differentiation of the male reproductive tract has been well established both in vivo and in vitro experiments (Guraya, 1980). Several studies have indicated that the fetal testes are able to synthesize steroid hormones at the time of sexual differentiation (Ferguson, 1965). The secretion of androgen by the fetal testes has been regarded as the primary determinant of male phenotypic sexual

differentiation (Jost, 1973). The activity of HSD has been demonstrated in the Leydig cells of the fetal testes in several mammalian species (mouse: Hitzeman, 1962; rat: Levy et al., 1959; Schlegel et al., 1967; rabbit: Wattenberg, 1958; Goldman, 1972).

As far as the ovary is concerned, two types of ovarian development are recognized (section 1.3): those showing immediate or delayed meiosis. The two kinds of ovaries differ in the time at which sex steroid synthesis begins (Grinsted, 1981). In those species with delayed meiosis, the ovaries secrete high amounts of oestrogen prior to the onset of meiosis. On the other hand, little or no steroids are produced in the ovaries of those species with immediate meiosis at this time (Byskov, 1986). The early development of steroid secreting cells in the ovaries of species with delayed meiosis has been attributed to the presence of germ cell compartments, that is, female sex cords (Byskov, 1986). The latter suggests that these compartments are necessary for the differentiation of steroid secreting cells and the establishment of hormone production. The ovaries of those species with delayed meiosis are comparable with male gonads in as much as the germ cells are compartmentalized. Thus, the observation of HSD activity in the rat testis as early as day 15 of gestation, the time when sex cords are formed, and its absence in the ovaries before day 9 post partum (Schlegel et al., 1967) may be attributed to the presence of germ cell compartments.

Numerous studies exist to localize the presence of HSD in sections of ovarian tissues (mouse: Ferguson, 1965; Hart & Baillie, 1966; Hadjioloff et al., 1973; Muller, 1975; Hoyer & Byskov, 1981; rat: Levy et al., 1959; Presl et al., 1960; Guraya, 1964b; Pupkin et al., 1966; Schlegel et al., 1967; Goldman & Kohn, 1970; Hoyer & Andersen, 1977; hamster: Guraya, 1965; rabbit: Wattenberg, 1958; Goldman et al., 1972, Guraya, 1967; Guraya, 1968; George et al., 1979;

Grinsted, 1982; cattle: Guraya, 1968; human: Goldberge et al., 1963; Fienberg & Cohn, 1965; Baillie et al., 1966; Goldman, 1966; Jones et al., 1968; Motta et al., 1970; Nagai, 1985).

HSD activity has been localized in various ovarian tissues. Most authors detect HSD in the interstitial tissue cells (see Table 4.2). Other ovarian tissues such as the granulosa cells, theca interna, granulosa lutein cells, atritic follicles, hilar cells and intraovarian rete cells, have also been regarded as sites for steroid synthesis (see Table 4.2).

Although HSD has been studied intensively in the ovaries of several mammalian species, no work in this area has been done on marsupials. In this chapter, an attempt will be made to demonstrate HSD enzyme in potoroo and Bennett's wallaby pouch young and the results will be correlated with light and electron microscopical observations (described in chapter 3).

As mentioned in Section 1.3, Byskov and Lintern-Moore (1973) distinguish three parts of the rete system in the mouse: the extraovarian rete, the connecting rete and the intraovarian rete. It was observed that in neonate mouse ovaries, only the intraovarian rete showed positive HSD activity (Hoyer & Byskov, 1981). These authors suggest that the interaction between germ cells and the intraovarian rete cells triggers the activity of HSD in the latter.

Hart et al (1966) report fluctuations in the HSD activity of granulosa cells in different follicles. Some granulosa cells showed either a negative or a uniform reaction while in the third group a strong reaction was detected in the peripheral granulosa cells only.

Similar regional differences were observed in HSD reaction in the granulosa cells by Hoyer and Byskov (1981) in the mouse and Hoyer and Andersen (1977) in the rat. The granulosa cells of centrally located

follicles (type 3b and 4, see Section 3.3) in mouse ovaries showed similar HSD activity to the intraovarian rete cell cords with which they were in contact (Hoyer & Byskov, 1981). They observed that four days later, some type 4 follicles showed negative HSD activity and subsequently when types 5, 6 and 7 follicles appeared, they showed differences in HSD activity as well. Hoyer and Byskov (1981) found that those follicle types 5, 6 and 7 which lost their connection to the intraovarian rete showed positive reaction in the peripheral granulosa cells only.

The ultrastructural features of both the granulosa cells and the interstitial gland cells indicate their involvement in steroid synthesis (Grinsted, 1981; Hadjioleff et al., 1983). They consist of clusters of lipid droplets associated with ribosomes, well developed Golgi complex, granular endoplasmic reticulum and mitochondria with tubular cristae.

It has been observed that HSD synthesis in fetal pig ovaries occurs earlier than the development of ultrastructural features related to steroidogenesis (Scheib & Lombard, 1972; Pelliniemi, 1976). The latter found that all the cell types of pig ovaries at 24 days post coitum lacked organelles related to steroidogenesis, although Moon & Raeside (1972) demonstrated HSD in the mesenchyme of the indifferent gonads at the same stage of development (quoted by Pelliniemi, 1976).

It was found that the activity of NADH diaphorase is high in all ovarian tissues of the mouse irrespective of the absence of HSD activity (Hoyer & Byskov, 1981). This enzyme catalyses the reduction of the tetrazolium salt by pyridine nucleotide, thus producing the colour product of the HSD reaction.

Jacoby (1962) reports that the only substrates which can be utilized by HSD enzyme in tissue sections are dehydroepiandrosterone

and pregnenolone. Moreover, Mori and Matsumoto (1970) find that the activity of HSD is higher in tissue sections when dehydroepiandrosterone is used as a substrate.

4.1.2. DESCRIPTION AND DISTRIBUTION OF MACROPUS RUFOGRISEUS

Bennett's wallaby is a common animal in zoos since it breeds readily in captivity (Tyndale-Biscoe, 1975). It is found in eastern and south eastern Australia and Tasmania, inhabiting woodland, forest edges and coastal scrub (Ride, 1970).

Bennett's wallaby is polyoestrous and monovular (Walker & Rose, 1981). It is a seasonal breeder and has a gestation length of 29.4 days and an oestrous cycle of 31.9 days (Tyndale-Biscoe, 1975).

4.2 MATERIALS AND METHODS

4.2.1 TISSUE PREPARATION

Immature (newborn-10 days old) and adult mice acting as controls and pouch young potoroos were killed by ether. One of the mouse and potoroo ovaries was removed quickly and placed either in a plastic mould or on a cork disc (3-4 mm thick) with OCT compound (a mounting edium for freezing tissue). The ovaries together with the surrounding medium were then immediately dropped for 2-3 seconds into graduated plastic beakers of 2-methyl butane (Isopentane), which had been cooled in liquid nitrogen. The tissues were then kept in a flask of liquid nitrogen before storing in a deep freeze at -70^{o}C.

Bennett's wallaby pouch young ovaries were obtained from the Wellcome Laboratories, London, already frozen in liquid nitrogen by Dr Ullmann, University of Glasgow.

The blocks were sectioned at 10μm in a cryostat maintained at -22^{o}C and the sections were attached to clean glass cover slips by transient thawing, 2-3 sections per cover slip.

4.2.2 RATIONALE FOR DEMONSTRATION OF DEHYDROGENASE

Dehydrogenase activity is demonstrated histochemically by substituting an artificial electron acceptor for the naturally occuring substances constituting an electron transport chain.

The artificial substance chosen is tetrazolium salt, which is colourless, water soluble and is able to accept the hydrogen removed from the substrate.

Tetrazolium salt which has been reduced in this way produces an insoluble, highly coloured microcrystalline deposit of a formazan compound at the site of reaction.

Unfixed frozen sections are incubated in medium containing the specific substrate, the tetrazolium salt, phosphate buffer (pH 7.4) and coenzyme NAD (Nicotinamide adenine dinucleotide).

The tetrazolium salt in the incubation medium competes with the naturally occuring electron carriers of the cell. In order to direct electrons from the oxidized substrates to the tetrazolium salt i.e. inhibit the flow of electrons to oxygen, potassium cyanide is added to the incubation medium.

For the localization of Δ 5, 3β hydroxysteroid dehydrogenase (HSD), the physiological substrates used were dehydroepiandrosterone (DHA) and pregnenolone.

4.2.3 RATIONALE FOR DEMONSTRATION OF DIAPHORASE

When the tetrazolium salt is reduced by pyridine nucleotide (NADH or NADHP), the reaction is catalysed by an enzyme either NADH diaphorase or NADPH diaphorase. Thus the coloured product of a histochemical method for coenzyme - linked dehydogenase is formed by the catalytic action of another enzyme, the diaphorase.

$$NAD^+ \xrightarrow{\Delta 5,\ 3\beta\ HSD} NADH$$

NAD$^+$ oxidized coenzyme

NADH reduced coenzyme

$$\text{Tetrazolium salt} + \text{NADH} \xrightarrow{\text{NADH diaphorase}} \text{formazan} + NAD^+$$

Tetrazolium salt (H acceptor) + NADH (acts as a substrate) → formazan (reduced form) + NAD$^+$

Pyridine nucleotides are freely diffusible, so localization of HSD requires that diaphorase be present also in the cells. It is necessary therefore to demonstrate the distribution of diaphorases in order to get accurate localization of a coenzyme-linked dehydrogenase.

4.2.4. HISTOCHEMICAL TECHNIQUE FOR DEMONSTRATION OF HSD

Frozen unfixed sections were processed as follows:-

(1) Place in cold acetone (4^{o}C) for 2 mins, to extract the lipid droplets.

(2) Wash in phosphate buffer (4^{o}C) for 2mins, to remove water soluble endogenous substrates.

(3) Add a few drops of medium to the sections, then cover with a petri dish to prevent evaporation of the medium and incubate at 37^{o}C for 45 mins to 1 hour.

(4) The 5 incubation media A-E (see Table 4.1) were made up and filtered immediately before use.

(5) After incubation, rinse sections in distilled water.

(6) Fix in 4% formaldehyde or in 10% formol saline for 1 hour.

(7) Rinse in distilled water.

(8) Mount in neutral glycerine jelly (aqueous mountant).

The sections were then examined using a Wild M20 microscope and photographed using panatomic film.

Table 4.1

Incubation	Stock Solution	Substrate DHA in DMF	Substrate pregnenolane	Coenzyme NAD	Coenzyme NADH	Water	DMF	Enzyme to be demonstrated
A	x	x		x				HSD
B	x		x	x				HSD
C	x			x		x		Control
D	x			x			x	Control
E	x				x			diaphorase

DHA = dehydroepiandrosterone

DMF = dimethyl-formamide (to dissolve the substrate)

stock solution = nitroblue tetrazolium (NBT) + phosphate buffer + potassium cyanide + + magnesium chloride + polyvinyl pyrollidone

4.3. RESULTS

Δ5 3β Hydroxysteroid dehydrogenase (HSD) was demonstrated in the ovaries of adult mice _ but the immature ovaries of mice (newborn-up to 10 days old), Bennett's wallaby (age 42 to 140 days) and potoroo pouch young (newborn and 86 days old) gave a negative reaction.

Difficulties were encountered during the localization of HSD enzyme, since it is loosely bound to the tissues. A stabilizer, polyvinyl pyrollidone (PVP), which is a polymer was added to the incubation media. PVP prevents the loss of this enzyme from frozen unfixed sections without interfering with HSD activity (Lojda et al., 1979). Various concentrations were tried out and 20% found to give satisfactory results with mouse ovaries.

In the mouse, a strong reaction was observed in the theca layer as well as in the interstitial gland tissue while the granulosa cells showed negative or a very weak reaction (fig. 4.1)

Both dehydroepiandrosterone and pregnenolone substrates gave positive reactions, that is, they were both utilized by HSD enzyme in the ovaries of adult mice.

The time taken for a colour reaction to develop, which was visible to the naked eye, was about 45 minutes in medium containing dehydroepiandrosterone as a substrate and about 60 minutes where pregnenolone was used as a substrate. The intensity of the reaction product remained unchanged even after incubating the section in the above substrates for a longer time, that is 2-3 hours.

Sections of ovaries which were used as controls for HSD enzyme activity, that is incubated in medium with either dimethyl formamide or water but without dehydroepiandrosterone or pregnenolone substrates (Table 4.1) were devoid of formazan deposit.

Activity of NADH diaphorase enzyme was high in the ovaries of the

three species used (see Fig. 4.2 - Fig. 4.6) inspite of the absence of HSD in the marsupials pouch young. In the case of the oldest Bennett's wallaby pouch young, the NADH diaphorase reaction was uneven, with the granulosa of the centrally located follicles staining dark blue while the remaining tissues showed a purple colouration.

Abbreviations

f	follicle
fl	fibrous layer
g	granulosa cell
it	interstitial tissue
m	mesothelium
mc	medullary cord
o	oocyte
t	theca layer

Fig. 4.1a. Transverse section through a portion of the ovary of an adult mouse. Note the strong hydroxy steroid dehydrogenase reaction in the interstitial tissue and the theca layer and the weak reaction in the granulosa cells following incubation in pregnenolone. (Bar = 50 μm).

Fig. 4.1b. Detail of marked region in Fig. 4.1a. (Bar = 25 μm).

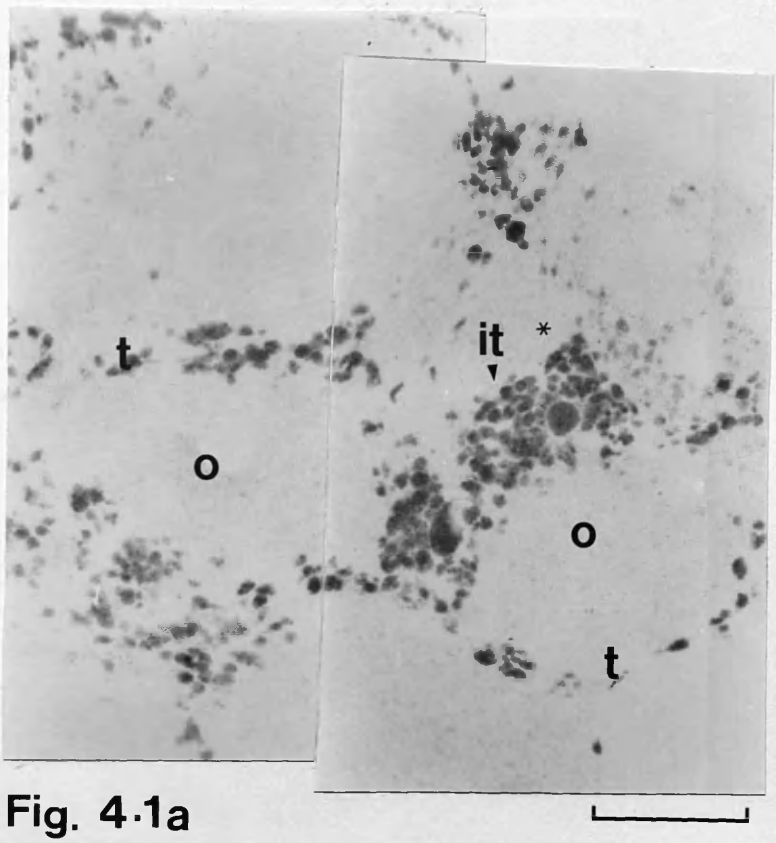


Fig. 4.1a

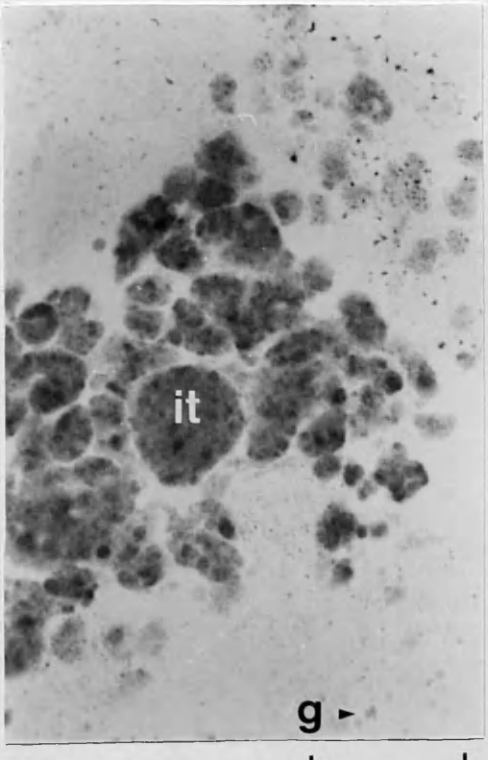


Fig. 4.1b

Fig. 4.2a. Portion of a frozen unfixed section of the cortex of the ovary of an 86 day old pouch young potoroo, stained with haematoxylin and eosin. (Bar = 50 μm).

Fig. 4.2b. Same region as shown in Fig. 4.2a, showing NADH diaphorase activity in the somatic cells. The oocytes remain unstained. (Bar = 50 μm).

Fig. 4.2c. Same ovary as in Fig. 4.2a, through the medulla, showing NADH diaphorase activity in the medullary cords. (Bar = 50 μm).

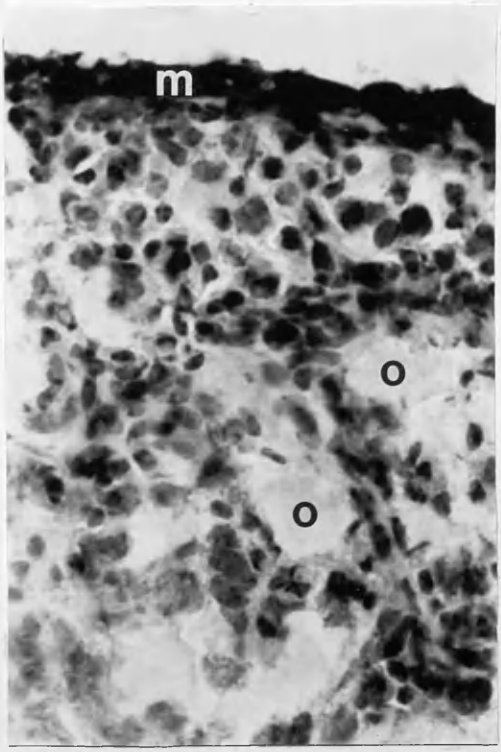


Fig. 4.2a

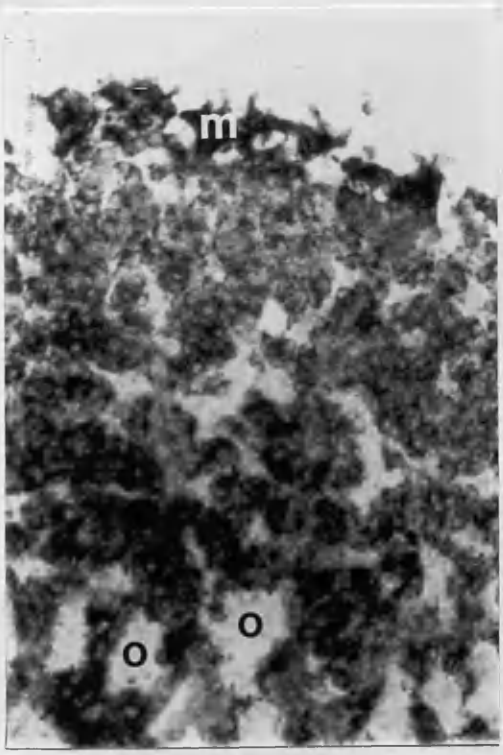


Fig. 4.2b

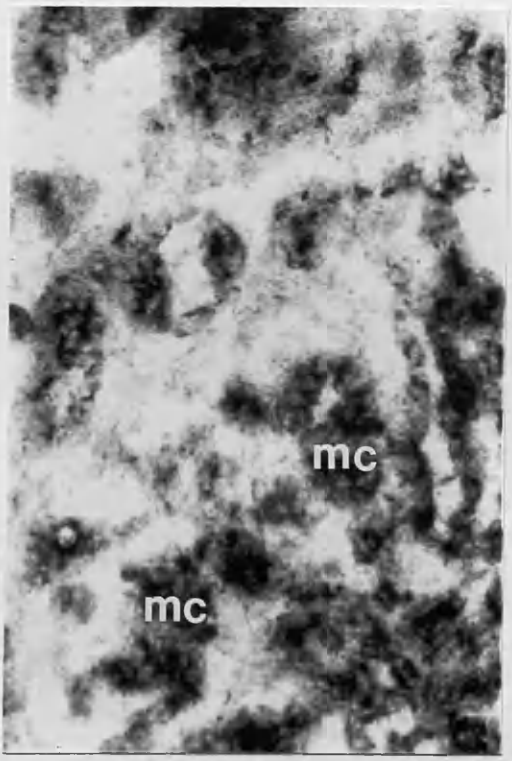


Fig. 4.2c

Fig. 4.3. Transverse section of a portion of the ovary of a 6-week old pouch young Bennett's wallaby. Note strong NADH diaphorase activity in the somatic cells only. (Bar = 50 μm).

Fig. 4.4a. Transverse section of a portion of an ovary of a 7 week old pouch young Bennett's wallaby, showing NADH diaphorase activity in the somatic cells of the cortex. (Bar = 50 μm).

Fig. 4.4b. Same ovary as in Fig. 4.4a to show the strong NADH reaction in the medullary cords and the weak activity in the fibrous layer between the cortex and the medulla. (Bar = 50 μm).

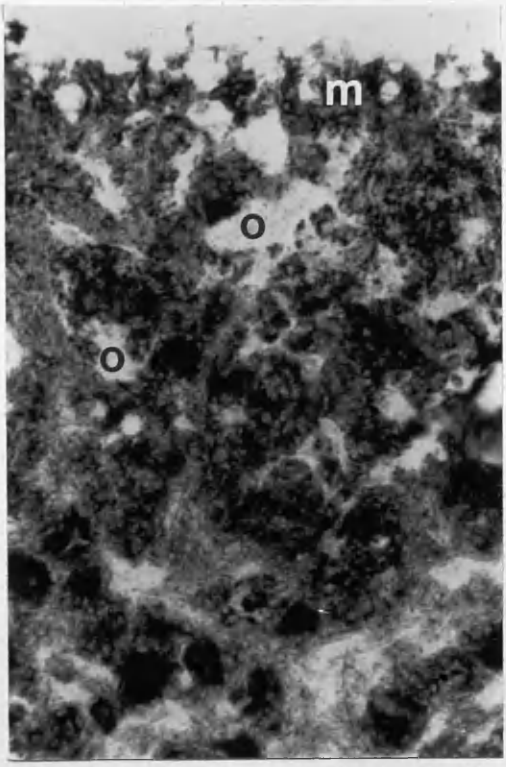


Fig. 4·3

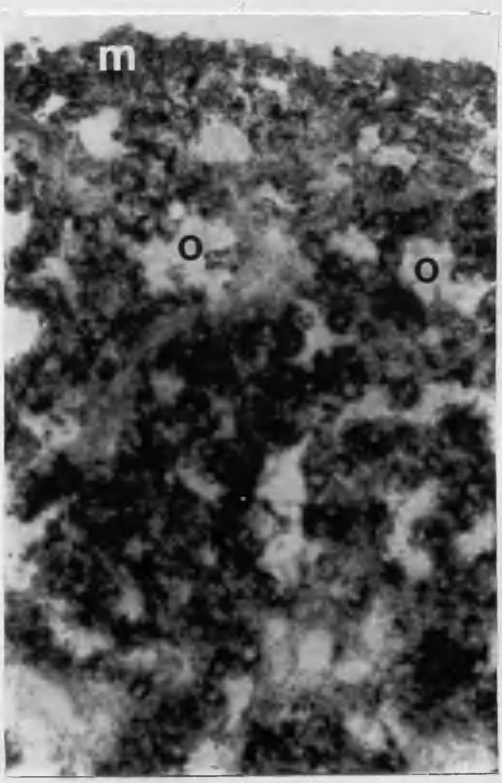


Fig. 4·4 a

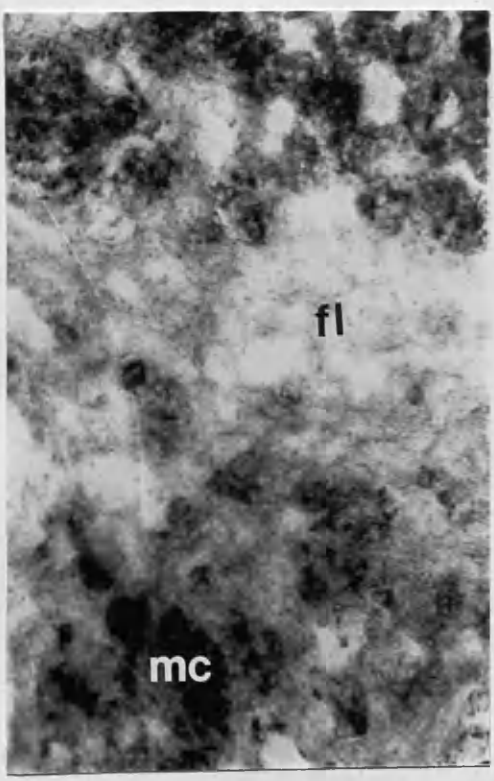


Fig. 4·4 b

Fig. 4.5. Transverse section through a portion of the ovarian cortex of an 8 week old pouch young Bennett's wallaby, showing NADH diaphorase activity in the somatic cells only. (Bar = 50 μm).

Fig. 4.6a. Transverse section through part of the ovary of 20 week old pouch young Bennett's wallaby, stained with haematoxylin and eosin. (Bar = 50 μm).

Fig. 4.6b. Same region as in Fig. 4.6a, to show NADH diaphorase reaction in the somatic cells. The oocytes remain unstained. (Bar = 25 μm).

4.4. DISCUSSION

The results presented in this study regarding the appearance of $\Delta 5$ 3β hydroxysteroid dehydrogenase enzyme (HSD) in the ovaries of adult mice are comparable to those reported previously for this species (see p. 185).

The observations, however, indicate that HSD activity is absent from all the immature stages studied, in both marsupials (potoroos: newborn and 86 days old; Bennett's wallabies: 41-140 day old pouch young) and mice (new born - 10 days old).

Potoroo ovaries, like those of mice, belong to the type characterized by immediate meiosis (see chapter 3). In ovaries of this type, little or no steroids are secreted prior to folliculogenesis and this observation appears to be related to the absence of germ cell compartments or sex cords (Byskov, 1986).

In ovaries of the delayed meiosis type (Grinsted, 1981) the sex cords form prior to the onset of meiosis and, according to Byskov (1986), this is a prerequisit for the differentiation of steroid secreting cells and hormone synthesis. Thus the negative activity noted in the macropod marsupial ovaries used is not suprising since these species lack germ cell cords and meiosis initiates at early stages of ovarian development.

As shown in Table 4.2, Hart et al (1966) observed weak HSD reaction in mouse ovaries at day 12 after birth. They report that prior to the third week of life the granulosa cells of most ovarian follicles show an even distribution of HSD enzyme. Subsequently the pattern of activity changes with the granulosa of some follicles showing no activity, that of others having an even distribution of HSD while in the third group the granulosa of the peripheral layers exhibit higher intensity of reaction than those of the inner part.

Müller (1975), in contrast, found no trace of HSD enzyme activity in mouse ovaries prior to day 14 of life. At this stage, however, he observed strong reaction deposit in the interstitial tissue while the granulosa cells exhibited a very weak reaction. At later stages of ovarian development, that is at five weeks of age, the theca layer surrounding the follicles as well as the corpora lutea showed a strong positive reaction (see table 4.2).

Hajioloff et al (1973) detected HSD activity in neonate to 3 week old mouse ovaries. They state that while the granulosa of new born and immature mice (1 - 15 day post partum) demonstrates strong activity, that of adult animals shows only a moderate activity: the granulosa of atretic and some preovulatory follicles shows a positive reaction while those of developing follicles exhibit a negative reaction. In the preovulatory follicles, these authors found that HSD reaction was generally present only in granulosa cells attached to the basement membrane. In both new born and mature animals, the interstitial tissue showed a positive reaction.

Hajioloff et al (1973) found that although the granulosa cells showed a positive HSD reaction in the neonates, their ultrastructural features did not reveal an involvement in steroid synthesis: except for possesing lipid droplets associated with ribosomes, the granulosa cells did not have any other organelles related to steroid synthesis. The granulosa cells of preovulatory follicles, on the other hand, were characterized by the presence of such organelles and many lysosomes. They interpret the presence of these features in the granulosa cells of preovulatory follicles as signs of steroidogenic activity related to the precocious luteinization of granulosa cells. The presence of lipid droplets in the granulosa cells of neonate ovaries is considered as an early sign of atresia. They argue for their interpretation by stating that numerous atretic follicles are present in the sections of

prepubertal ovaries.

As mentioned in section 4.1, the presence of HSD enzyme in tissue sections is considered as an indication of steroidogenic capability (Guraya, 1977). However, the results of Hajioloff et al (1973) presented above suggest that the presence of HSD in neonate ovaries does not necessarily mean that the latter are capable of producing steroid hormones. These observations thus suggest that the method used by many authors for the demonstration of HSD is not reliable for testing for the presence of steroid hormones. Moreover, these authors' results raise the possibility that the presence of HSD enzyme does not need the elaborate cellular machinery required for hormone production.

Pehleman and Lombard (1978) studied the ultrastructure of ovarian interstitial tissue in embryonic to 3 day old mice and report similar observations to those of Hajioloff et al (1973). They found no sign of the morphological machinery for steroidogenic activity in mouse ovaries prior to day 3 post partum: at this stage only lipid droplets and occasional membranes of smooth endoplasmic reticulum were noted. Small interstitial gland cells with fully differentiated tubular mitochondria and sparse endoplasmic reticulum were first observed on day 9 after birth. Fully differentiated interstitial gland cells were present by day 14 post partum.

Hoyer and Byskov (1981), also working on the mouse, detected HSD activity at day 7 after birth. At this stage they report that only the rete cells, which have an intraovarian position close to the oocytes (according to them, the intraovarian rete), and the granulosa cells of growing follicles develop HSD activity. They found that the activity of the granulosa cells varied with the type of follicle and with age. They observed that on day 7 after birth both the granulosa cells of the centrally located follicles (Type 3b-4 follicles) and the intraovarian rete cell cords, with which they are in contact, have the

same intensity of reaction. Moreover, on day 11, Hoyer and Byskov (1981) found that some type 4 follicles did not show any HSD reaction. They also report that on days 15 and 22 a difference in HSD activity was found among the large centrally placed follicles (type 5,6,7). In those follicles which according to them lost their connection to the intraovarian rete, HSD intensity decreases and is confined to the peripheral granulosa cells. On the other hand, in those follicles which are connected to the intraovarian rete, the granulosa cells exhibit a uniform HSD reaction. The intraovarian rete subsequent to losing its connection with the newly formed follicles becomes transformed into interstitial tissue and shows a strong HSD reaction.

Hoyer and Byskov (1981) interpret their observations of regional differences in the granulosa cells of large follicles in the following way: the follicles of the first category (with localized HSD reaction) are healthy and may be able to ovulate while those of the second category (showing uniform HSD reaction) may be atretic and become reverted to the intraovarian rete compartment.

The preliminary observations presented in this chapter on the first appearance of HSD enzyme in mouse support those of Müller (1975). There was no trace of HSD reaction in the newly born, 5 day and 10 day old animals. The discrepancy in the first appearance of HSD between different authors working on the same species could be due to differences in the strains of mice used; or to the subjective visual evaluation of the intensity of reaction as suggested by Hoyer & Byskov (1981).

Different results have been reported for different species regarding the site of HSD reaction in adult ovarian tissues. While most investigators detected HSD activity in the interstitial tissue (see Table 4.2), Fienburg and Cohn (1965) working on human and Hoyer and Byskov (1981) examining mouse ovaries, detected this enzyme also

in the hilar cells and in the intraovarian rete respectively.

As far as the granulosa cells are concerned, most investigators report their positive HSD reaction (Table 4.2). However, according to some authors HSD is absent from the granulosa cells of adult ovaries of the mouse (Ferguson, 1965), rat (Jacoby, 1962), rabbit (Wattenberg, 1958; Goldman, 1972; Mori & Matsumoto, 1970) and pig (Bjersing, 1969).

In this study HSD reaction was detected in the interstitial tissue, granulosa cells and theca layer of adult mouse ovaries. The results presented in this chapter regarding the site of HSD reaction in adult mouse ovaries are in agreement with those reported for the mouse (Hajioloff et al., 1973) and rat (Schlegel et al., 1967; Hoyer & Anderson, 1977). Besides the three sites mentioned above for HSD activity, Hart et al (1966) and Müller (1975) found that the lutein cells exhibit reaction deposit. The intensity of the reaction observed in this study is comparable to those reported by Müller (1975): a strong HSD reaction was detected in the theca layer as well as in the interstitial tissue while the granulosa cells showed a very weak reaction deposit.

The only observations of HSD enzyme in the intraovarian rete, which is present at the juvenile stages, are those of Hoyer and Byskov (1981), working on 1-22 day old immature mouse ovaries. These authors do not report the presence of HSD in the interstitial tissue which has been reported by Hart et al. (1966), Hadjioloff et al. (1973) and Müller (1975) for the same species. However, it is possible that they have misinterpreted the interstitial tissue for the rete cells.

The negative reaction for HSD in the ovaries of the macropods used in this study is not due to the absence of NAD diaphorase enzyme. It was observed that the activity of the latter was high in all the ovaries studied, namely, mice, potoroos and Bennett's wallabies. In the older Bennett's wallaby pouch young, that is 140 days old, the

activity was uneven in the ovary; the granulosa cells of the centrally located follicles stained dark blue while the remaining tissues showed a purple colour.

The apparent absence of HSD enzyme may be due to its presence in the ovaries at a very low level which cannot be detected by histochemical methods. Negative reaction for HSD was obtained even after incubating the macropod ovaries in the substrates for 2-4 hours. The prolonged incubation of the ovarian tissues in pregnenolone and dehydroepiandrosterone resulted in the appearance of some faint colouration visible to the naked eye which at first gave the impression of a positive HSD reaction. Careful reinvestigation of ovarian sections, however, indicated that this was an artifact and it occurred as a result of a non-specific reaction.

As mentioned earlier, the absence of HSD activity in the macropod marsupials listed above confirms the observation that steroid synthesis does not become established prior to folliculogenesis in those species with immediate meiosis. Morever, it was found that although some of the medullary cords were transformed into interstitial tissue cells in an 86 day old pouch young potoroo (see p. 90), they were not highly differentiated and lacked the characteristic features of mature steroid secreting cells. Electron micrographs indicate the presence of only sparse smooth endoplasmic reticulum in the cytoplasm of the interstitial tissue and few round or rod shaped mitochondria. However, large lipid droplets are present.

This is the first attempt to demonstrate HSD enzyme in marsupial gonads. Although in this study positive results were not obtained for reasons suggested previously, further work is expected to demonstrate HSD enzyme in the ovaries of older marsupials which have undergone folliculogenesis.

Table 4.2. Demonstration of HSD in different cell types in mammals

Authors	Species	Age	Granulosa cells	Interstitial gland cells	Lutein cells	Theca interna	Theca externa	Hilar cells	Intraovarian rete cells	Stroma	Atrctic follicles	Mesothelium
Jacoby (1962)	Mouse			*								
Ferguson (1965)	"	Adult	+	++++			+			+		
Hart et al (1966)	"	1-4 wks	*	*								
	"	5 wks	*			*						
	"	6-10 wks	*	*	*	*						
Schiebler & Lombard (1972)	"		*									
Hadjiloff et al. (1973)	"	1 day	++++	++								+++
	"	4 day	++++	+++								++
	"	10 & 12 day	+++	+++		++++						++
	"	15 day	++++	+++		++++						+
		mature (4 mths)	++	+++		+++						
Muller (1975)	"	Newborn + 1 wk	no activity									
	"	2 & 3 wks	+	++++								
	"	5 wks & 4 mths	+	++++	++++	++++						
Hoyer & Byskov (1981)	"	1 & 4 day	no activity									
	"	7, 11	*					*				
	"	15,22 day										
Wattenberg (1958)	rat	adult		*	*							
Jacoby (1962)	"			*	*							
Prest et al (1965)	"	8,10 day		*		*						
Schlegel et al (1967)	"	9 day		++								
	"	10 day		++++		++++						
	"	14 day	++	++++		++++						
Goldman & Cohn (1970)	"			*		*						
Hoyer & Anderson (1977)	"			*		*						
Wattenberg (1958)	rabbit	adult		*								
Goldman (1972)	"	29 post coitum		*								
Jacoby (1967)	"			*								
Mori & Matsumoto (1970)	"			*								
Bjersing (1969)	pig		*		*	*						
Fienburg & Cohn (1968)	human				++++	++++						
Motta et al (1970)	"		*									
Goldman et al (1966)	"		*									

* = strength of reaction not indicated; + = very weak reaction; ++ = weak reaction; +++ = moderate reaction; ++++ = strong reaction

CHAPTER FIVE

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Since individual sections include discussion on the work undertaken, this chapter brings together the general conclusions drawn from this investigation and makes recommendation for future study.

The major aim of this study was to compare the process of gonadogenesis in eutherian and marsupial mammals and to throw light on some problems - for instance, the origins of the blastema, granulosa cells, interstitial tissue (IT), polyovular follicles (POFs), and the first appearance of hydroxysteroid dehydrogenase enzyme (HSD) in the ovaries of macropods.

The observations made here regarding the origin of the blastema in the mouse indicate that it arises from the mesothelium and the mesonephric tubules, as postulated by Wartenberg (1983). In the macropod pouch young studied the blastema also has a dual origin, but from the mesothelium and the mesonephric mesenchyme. The non-involvement of the mesonephric tubules in macropod gonad formation may be accounted for by the fact that in these animals the mesonephric tubules are well developed and functional. In mouse embryos they are poorly developed and rudimentary and contribute cells to the gonads.

The observations reported in this study suggest that gonadal sexual differentiation in the mouse, tammar wallaby and the bettong occurs as a result of "autodifferentiation" of the blastema cells. A similar pattern of gonadal sexual differentiation has been reported for the rat (Jost, 1973) bandicoot and brush-tailed possum (Ullmann, 1981a, 1984b). In this study it was found that at day 12 of gestation in the mouse (p. 42) and day 2 and day 3 of pouch life in the bettong and the tammar wallaby respectively, the large blastema cells (pre-Sertoli cells) swell, clump together, exhibit low affinity for the stains used and enclose germ cells forming sex cords.

Mouse and tammar wallaby ovaries were reinvestigated between 12-14 days post coitum and 9-18 days of pouch life respectively; while ovary development is described for the first time in the potoroo (from day 1 to adult stages) and the bettong (day 1-59).

Since comparable stages of ovary development in mouse embryos were not reinvestigated in this study, the structure of the macropod ovaries at later stages of development was compared to those reported in the literature for other eutherian and marsupial mammals.

Two kinds of ovarian development have been described for mammals: those with immediate and those with delayed meiosis (see Section 3.1). Both mouse and the macropod ovaries reported on here belong to the first category of animals. However, the observations presented above indicate that the mitotic and meiotic activities of female germ cells in the former are closely synchronized while those of the latter are not. The presence of asynchrony of divisions has, however, been reported for some eutherian mammals (ferret: Deanesly, 1974; human: Baker, 1963).

The two kinds of ovarian development (that is with immediate or delayed meiosis) deviate from each other also in the time at which sex steroid synthesis begins (Byskov, 1986). No significant sex steroids are produced by the newly differentiated ovaries which are characterized by immediate meiosis while those ovaries with delayed meiosis secrete a high amount of oestrogen. The absence of hydroxy-steroid dehydrogenase enzyme (HSD) from the ovaries of the macropods used here (the potoroo and Bennett's wallaby) confirms the observation that steroid synthesis does not become established until folliculogenesis in those species with immediate meiosis. Further support for this contention is deduced from ultrastructural studies on the ovaries of the potoroo at the same stage of development. Electron micrographs reveal that although the presence of a transitory stage in

the development of IT is observed at day 66-69 of pouch life (chronological age of 86 days), it is not highly differentiated and lacks the characteristic features of steroid secreting cells (see Section 3.4).

The observations regarding the origin of the IT in the potoroo indicate that these form as a result of the differentiation of the medullary cords (which have a blastemal origin) in the centre of the ovaries.

The mode and extent of rete development differs in different mammals. In some eutherian and all marsupial species investigated (ferret: Deanesly, 1975; tammar wallaby: Alcorn, 1975; native cat: Fraser, 1919; Ullmann, 1984b; potoroo: this study) the rete arises from a condensation of cells at the anterior end of the gonadal rudiment. In other species (mouse: Byskov & Lintern-Moore, 1973; rat: Byskov, 1975; Stein & Anderson, 1979; cat and mink: Byskov, 1975) it derives from the mesonephros.

The extent to which the rete develops within the ovaries in different marsupials is variable. While in bandicoots the rete ends blindly at the hilar region (Ullmann, 1984a), it penetrates very deeply into the ovaries of the brush-tailed possum (Ullmann, 1986), the tammar wallaby (Alcorn, 1975) and the bettongs and potoroos studied here.

The histological work suggests that the granulosa cells in the potoroo and bettong arise from the medullary cords rather than from the rete and the findings are thus in agreement with those reported for a number of eutherian (ferret: Deanesly, 1970; guinea pig and rabbit: Deanesly, 1975) and marsupial (tammar wallaby: Alcorn, 1975; bandicoot: Ullmann, 1984a) mammals. The results, however, are at variance with the findings of other authors who derive the granulosa

from the rete in the mouse (Byskov & Lintern-Moore, 1973), rat (Stein & Anderson, 1979) cat and mink (Byskov, 1975).

According to the observations reported in this study, the POFs in the potoroo derive from isolated oocytes which become surrounded by a common envelope of granulosa cells, a condition postulated for some eutherian mammals (Mossman & Duke, 1973; Tagami & Akimoto, 1978).

The quantitative results show that there is a steady increase in the volume of potoroo ovaries with age. The volume of the ovaries increases from 0.014 mm^3 on day 5 post partum to 3.733908 mm^3 on day 120 and to 15.403465 mm^3 in adult ovaries. The observations presented in this study thus agree with those reported for eutherian mammals (rat: Beaumont & Mandl, 1962; guinea pig: Ioannou, 1964; cow: Erickson, 1966; rhesus monkey: Baker, 1966; and human: Baker, 1963) but differ from those suggested for the closely-related species, the tammar wallaby, by Alcorn (1975). The latter found that the ovarian volumetric growth curve is interrupted and is divided into 4 different stages (see p. 162).

This study leaves several questions for future work. It will be important to test some of the ideas on the origin of the different cell types presented above, particularly the origins of the granulosa cells, POFs and IT. This could be achieved by studying the fine structure of ovaries at the time of folliculogenesis to ascertain the presence of similarity between granulosa cells and medullary cord cells. It is also necessary to study the ultrastructure of ovaries with POFs to find out whether or not intercellular bridges are present between oocytes in POFs.

Since the presence of a transitory stage in the development of the IT was already observed at day 86 (equivalent to developmental age 66-69, see p. 90), it would be interesting to follow the further stages of this and therefore confirm the origin of IT from the

medullary cords.

Ultrastructural studies are needed to detect the time at which IT becomes fully differentiated. This work should be correlated with histochemical tests to demonstrate the first appearance of HSD enzyme, which is a necessary prerequisite for the production of sex steroid hormones.

It will also be important to confirm the suggestion presented above for macropods that the rete has no role in the formation of granulosa cells. This could be approached by culturing ovaries with or without the rete in order to find out if folliculogenesis occurs in the absence of the rete.

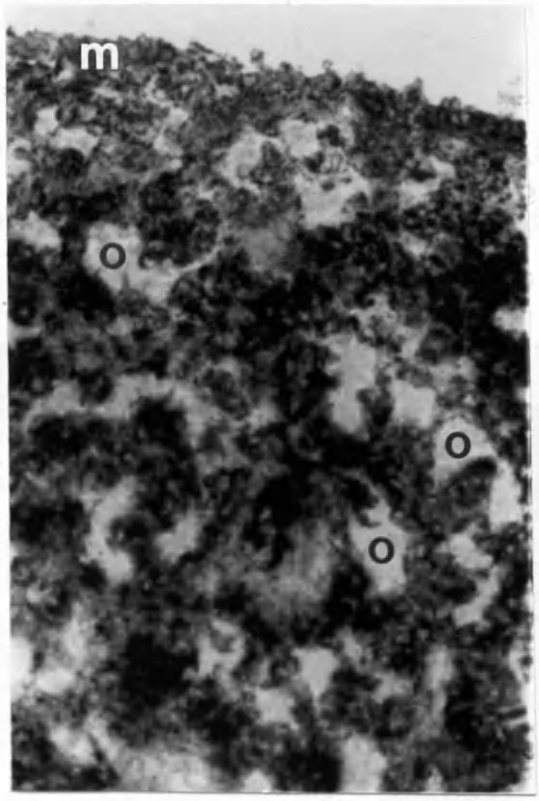


Fig. 4.5

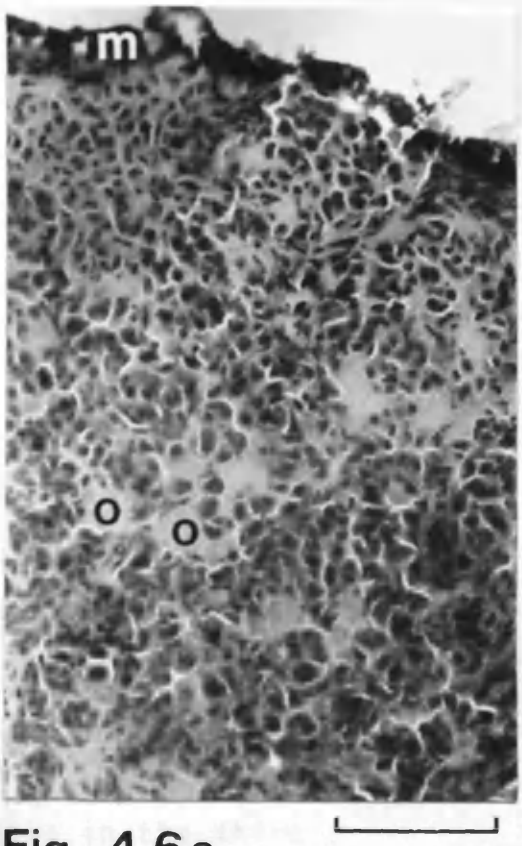


Fig. 4.6a

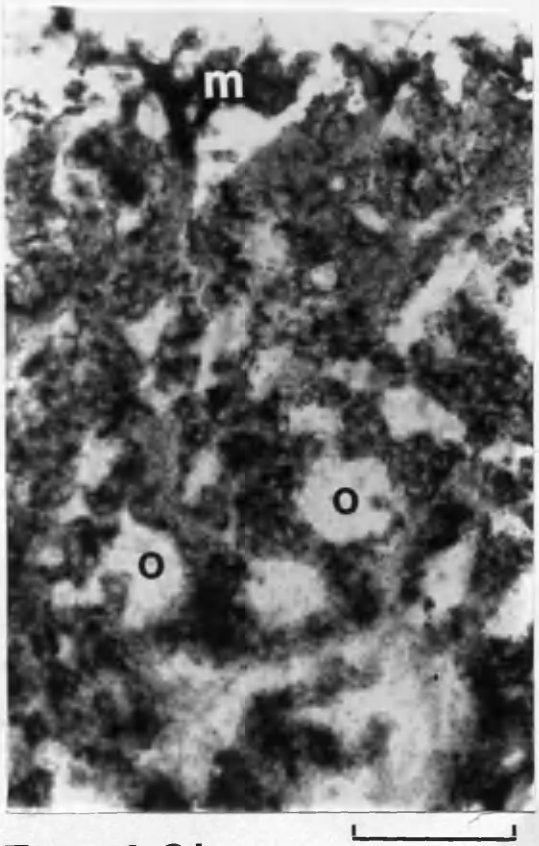


Fig. 4.6b

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APPENDIX A

Staining Techniques

A.1. Mayers Haemalum and Eosin:

1. Dewax sections in histosol for 5 minutes.
2. Dehydrate in 95%, 90%, 70%, 50% and 30% alcohols for 3 minutes in each.
3. Wash in water.
4. Stain with Haemalum for 6 minutes.
5. Rinse in water.
6. Rinse in Scotts' tap water till sections turn blue purple from a pink purple.
7. Rinse in water.
8. Dehydrate in 30%, 50%, 70%, 90%, 95% alcohol for 3 minutes in each.
9. Stain with eosin for 1.30 minutes.
10. Dehydrate in 95% alcohol for 1.30 minutes.
11. Clear in histosol for 5 minutes.
12. Mount in DPX.

A.2. Mallory's Triple Stain:

1. Dewax sections in histosol for 5 minutes.
2. Dehydrate in 95%, 90%, 70%, 50% and 30% alcohol for 3 minutes in each.
3. Wash in water.
4. Mordant in $Hgcl_2$ acetic acid for 10 minutes.
5. Rinse in water.
6. Stain with acid fuchsin for 15 seconds.
7. Differentiate in water for 20 seconds or longer.
8. Differentiate in phosphomolybdic acid for 60 seconds.

9. Wash in water.
10. Stain with Mallory for 2 minutes.
11. Wash in water for 10 seconds.
12. Differentiate in aniline blue 90% alcohol for 10 seconds.
13. Dehydrate in 2 changes of absolute alcohol for 10 seconds in each.
14. Clear in xylene.
15. Mount in DPX.

APPENDIX B

Solution Preparation

B.1. Karnovsky's Half Strength Fixative:

1. Dissolve 10 gm paraformaldehyde in 12.5ml of distilled water by heating to 60-70^{o}C and stirring on a magnetic stirrer.
2. Add a few drops of 1N NaoH stirring all the time till solution clears.
3. Cool solution and add 25ml of 25% gluteraldehyde solution.
4. Make up to 250ml with 0.2M sodium cacodylate buffer and add 0.125gm anhydrous calcium chloride.

B.2. 0.2 M Sodium Cacodylate Buffer:

1. Dissolve 2.14gm sodium cacodylate in 50ml distilled water.
2. Add 2.7ml of 0.2 M HcL.
3. Make up to 250ml with distilled water.

B.3. Veronal Acetate Buffer:

Dissolve 1.47gm sodium barbitone and 0.97gm hydrated sodium acetate in 50ml distilled water.

B.4. 1% Osmium Oxide Solution:

1. Dissolve 0.5gm osmium oxide crystals in 25ml distilled water and leave overnight.
2. Add the following solutions in order:
   5ml veronal acetate buffer, 5ml 0.2N HcL, and 15mL distilled water.

B.4. Gluteraldehyde Phosphate Fixative:

Add 10ml of 25% glutaraldehyde solution to a mixture made up of 38mL distilled water, 2ml 0.1% anhydrous calcium chloride solution and 50ml 0.2M phosphate buffer (pH = 7.4).

APPENDIX C

Pouch Young Measurements

C.1. Potorous tridactylus:

Animal Code Number	Age	Weight (gm)	Crown rump length (mm)	Head length (mm)	Manus (mm)	Pes (mm)	Tail (mm)	Ear (mm)
pt. 1	9 days							
pt. 2	11 "							
pt. 3	14 "							
pt. 4	1 "							
pt. 5	98 "	59						
pt. 6	120 "	61						
pt. 7	50 "	15.8						
pt. 8	70 "	30						
pt. 9	91 "	49						
pt.10	92 "	50						Folded back pigmented
pt.11	adult							
pt.12	90 days							
pt.13	89 "	39.28	97	45	17	38		
pt.21	adult (4 yrs)							
pt.27	9 yrs							
pt.30	adult							
pt.31	adult							
pt.32	adult (3.5 yrs)							
pt.33	adult (2.5 yrs)							
pt.36	adult (3.5 yrs)							
pt.38	86 days	81.4	100	44.45	40	40	80.3	1.95 back
pt.39	2.5 mths	45.3	85	43	20	42	82	Back

Animal Code Number	Age	Weight (gm)	Crown rump length (mm)	Head length (mm)	Manus (mm)	Pes (mm)	Tail (mm)	Ear (mm)
pt.40	adult							
pt.41	2-3 days	0.6753	10.9	8	2		5	none
pt.48	82 "		81.4	43.5	10.5	30.4	53	27.5
pt.49	50 "		51.0	21.0	5.0	10.0	16.0	6.0 back
pt.50	32 "		41.8	18.6	4.5	7.7	15.0	9.6
pt.51	48 "			46.8		38.0	63.0	17.5
pt.52	adult							
pt.53	"							
pt.54	8 days		18.3	8.7	2.3	3.6	5.1	
pt.55	1-3 days M	0.508	14.4	7.7	2.5	3.0	5.0	
pt.56	40-44 "		48.4	24.7	5.3	12.3	19.6	back
pt.57	1-2 "	0.451	15.4	17.5			5.3	
pt.58	75-79 "		72.0	42	10	30	53	
pt.60	100 "			52.5	12	48.5	82	22.0
pt.64	7-8 "		21.0	7.6	2.5	2.5	4.1	
pt.65	65-66 "		68.3	33.5	7.8	21.7	37.0	back
pt.67	117 "			43.0	10	28.3	48.4	15.0
pt.68	38 "		51.0	21.0	5	10.6	0.16	6.0
pt.69	75-76 "		81.5	44.5	9.5	34.3	60.0	
pt.70	29-30 "		45.0	20.0	4.6	9.2	9.2	17.5
pt.71	6-7 "		22.5	10.7	2.9	3.9	5.7	Just develope
pt.72	62 "		66.5	37.0	10	25	31	13.0
pt.73	25 "		43.0	19.4	4.8	9.6	13.5	back
pt.74	15 "		27.0	14.0	3.0	10.0	10.0	forwd
pt.78	adult (over 4 yrs)							
pt.81	70 days	14.61		25.86	7.90	16.66	31.38	8.84

Animal Code Number	Age	Weight (gm)	Crown rump length (mm)	Head length (mm)	Manus (mm)	Pes (mm)	Tail (mm)	Ear (mm)
pt.82	29 days							
pt.83	37 "							
pt.84	58 "	22.16	75.01	33.20	9.54	22.90	40.64	10.70
pt.85	35 "							
pt.86	38 "	9.99	54.82	24.48	5.88	13.52	27.78	7.52
pt.87	54 "							
pt.88	79 "	44.75	97.16	43.94	17.26	33.74	58.48	16.88
pt.89	63 "	26.69	82.82	35.90	9.82	23.84	49.78	11.96
pt.90	31 "							
pt.91	45 "	12.19	62.32	27.50	7.46	16.88	30.90	8.02
pt.92	38 "	9.29	55.92	24.30	5.38	12.82	25.01	6.40
pt.93	29 "			42.08	14.52	34.80	67.70	
pt.94	17 "	4		19.4		9		back
pt.97	30 "							

C.2. Bettongia gaimardi

Animal Code Number	Age (days)	Sex	Weight (gm)	Crown rump length (mm)	Head length (mm)	Manus (mm)	Pes (mm)	Tail (mm)	Ear (mm)
B 1	1 day	M		16.1	6.6			4.2	Just forming
B 2	2 "	M	0.461	14.9	7.8			5.0	
B 3	14 "	F		26.0	12.2	3.1	5.0	6.6	small pointing forward
B 4	15 "	F	2.75	31.0	14.3		6.7	6.3	-
B 5	7 "	F		20.7	10.5		4.1	6.0	
B 8	18 "	F		37.7	14.7	3.8	6.9	7.9	forward
B 9	42 "	F	15.9	55.7	21.5	6.3	20.0	29.0	8.0 backward
B10	28 "	F		42.0	20.0	4.7	12.4	21.0	6.5 backward
B11	84 "	F	106	112.0	47.0	10.0	66.0	104.0	
B12	59 "	F	40.0	80.6	38.0	8.5	35.0	50.4	13.0
B13	56 "	F			31.1				
B15	56 "	F		67.0	32.5	10.0	31.4	48.0	backward
B17	49.5 "	F		62.1	31.0	10.0	29.45	42.3	

C.3. Macropus eugenii pp = post partum: pc = post coitum

Animal Code Number	Age (days)	Sex
Me 1	5.5 (pp)	M
Me 4	24 (pc)	
Me 5	5 (pp)	M
Me 7	18 (pp)	F
Me 8	4.5(pp)	F
Me 9	6 (pp)	F
Me 11	6 (pp)	F
Me 12	24 (pc)	
Me 13	3 (pp)	F
Me 14	5 (pp)	F
Me 16	3 (pp)	F
Me 18	6 (pp)	M
Me 19	4 (pp)	F
Me 20	5 (pp)	F
Me 21	6 (pp)	F
Me 22	2 (pp)	
Me 23	8 (pp)	M
Me 26	5 (pp)	F
Me 27	3 (pp)	M
Me 28	24 (pc)	
Me 29	11 (pp)	F
Me 30	9 (pp)	F
Me 31	5 (pp)	M
Me 32	24 (pc)	
Me 33	24 (pc)	
Me 34	5 (pp)	F
Me 35	25 (pc)	

APPENDIX D

Estimation of Ovarian, Cortical and Medullary Volumes

D.1. Measurements of volume of ovary, cortex, and medulla of a 48-day old pouch young

Section No.	Profile	Perimeter (mm)	Area mm^3
1	ovary	0.000765	0.000044
	medulla	0.000	0.000
2	ovary	0.001968	0.000233
	medulla	0.000	0.000
3	ovary	0.002632	0.00040
	medulla	0.000	0.000
4	ovary	0.003120	0.000563
	medulla	0.000	0.000
5	ovary	0.003659	0.000798
	medulla	0.001758	0.000195
6	ovary	0.004003	0.000983
	medulla	0.001787	0.000203
7	ovary	0.004343	0.00124
	medulla	0.002208	0.000235
8	ovary	0.004739	0.001416
	medulla	0.002553	0.000383
9	ovary	0.005108	0.001644
	medulla	0.002643	0.000456
10	ovary	0.005156	0.001800
	medulla	0.002805	0.000417
11	ovary	0.005474	0.001877
	medulla	0.002701	0.00443
12	ovary	0.005282	0.001946
	medulla	0.002882	0.000540
13	ovary	0.005279	0.001952
	medulla	0.002710	0.000495
14	ovary	0.005485	0.001895
	medulla	0.003189	0.000512
15	ovary	0.005491	0.002057
	medulla	0.003392	0.000546

Section No.	Profile	Perimeter (mm)	Area mm^3
16	ovary	0.005511	0.002101
	medulla	0.003804	0.000608
17	ovary	0.005229	0.001917
	medulla	0.003605	0.00582
18	ovary	0.005151	0.001837
	medulla	0.003747	0.000550
19	ovary	0.004869	0.001670
	medulla	0.003051	0.000347
20	ovary	0.004698	0.001474
	medulla	0.003121	0.000569
21	ovary	0.004366	0.001367
	medulla	0.002817	0.000256
22	ovary	0.004270	0.001301
	medulla	0.000	0.000
23	ovary	0.0003967	0.00157
	medulla	0.000	0.000
24	ovary	0.0003573	0.000949
	medulla	0.000	0.000
25	ovary	0.003059	0.000677
	medulla	0.000	0.000
26	ovary	0.001527	0.000156
	medulla	0.000	0.000
27	ovary	0.000518	0.000020
	medulla	0.000	0.000

Volume of ovary = 1.992328 mm^3

Volume of medulla = 0.431210 mm^3

Volume of cortex = 1.992328 - 0.431210 = 1.561118 mm^3