GONADAL SEX DETERMINATION AND DIFFERENTIATION

IN RATS AND HAMSTERS

BY

WAI-SUM O

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Statement as to the author's participation in the work submitted

The composition of this thesis is that of the author. The author has acted as the principal investigator for these studies and has been personally involved in the design, organisation and conduct of the experiments. Contribution of technical assistance has been duly acknowledged.

Signed

Wai-Sum O

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PUBLICATIONS

The following publications have been produced in connection with this Thesis:

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ABSTRACT

Gonadal sex differentiation in the Liverpool Hooded strain of rat (Rattus norvegicus) and in the golden hamster (Mesocricetus auratus) was studied using organ culture and transplantation techniques.

It is known that when early fetal hamster ovaries (before Day 15 p.c.) are maintained in culture, the germ cells fail to undergo meiosis. In this study, it has been shown that when early fetal hamster ovaries (before Day 15 p.c.) were cultured together with rete ovarii, meiosis can occur, and proceed to the diplotene stage of meiotic prophase.

When fetal ovaries (Day 12-16 p.c.), with rete ovarii, were cultured in contact with fetal testes of the same age, normal ovarian development was undisturbed, but the male germ cells of the testis entered into meiosis precociously. These results suggest that meiosis in the ovary normally commences before meiosis in the testis because of some factor(s) associated with the presence of the rete ovarii.

Similar results were obtained in cultures of fetal testis separated from a fetal ovary with rete ovarii by a millipore filter (pore size 0.15 μm). The result suggests the diffusible nature of the meiotic inducing factor(s).

Rats were used in transplantation experiments. Fetal and neonatal rat gonads (Day 12 p.c. - Day 3 p.p.) were transplanted under the kidney capsule of intact or castrated adult hosts of both sexes. No difference was found between intact and castrated hosts of either sex in the subsequent sex differentiation of the grafts.

When male and female fetal gonads were transplanted together, testicular development was undisturbed. Ovarian development was unaffected after Day 20 p.c. while in the earlier ovaries, the ovarian organisation was altered in the following ways : retention of oogonia in the enlarged rete ovarii; ovarian tissue with groups of sex cords containing follicles with 3 to 6 layers of granulosa cells.

Both in vitro and in vivo culture techniques indicated that the germinal and somatic cells in gonadal differentiation may be under the influence of different controlling factors; this is the key to gonadal sex differentiation.

The interaction between germinal and somatic cells was also investigated using an 'artificial' chimaeric gonad, which contained female (XX) soma and male (XY) germinal cells. The somatic cells and germinal cells of fetal hamster gonads (Day 12 - 14 p.c.) can be separated from one another by trypsinisation, using 0.25% trypsin solution, followed by a plating technique. By recombining separated germinal and somatic cells of the opposite genotype, chimaeric gonads with XY germ cells surrounded by XX soma can be obtained and maintained in culture. The XY germ cells survive in the female somatic environment and behave like female germ cells in that they undergo precocious meiosis. In chimaeric gonads with XX germ cells surrounded by XY somatic cells, the female germ cells were unable to survive in the male environment.

These results indicate that both germinal and somatic cells in the gonad play a decisive role in the process of sexual differentiation. The XY germ cells can be transformed into 'oocytes' in an ovarian environment, but the XX germ cells cannot be transformed into spermatocytes by being in a testicular environment.

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Chapter I

INTRODUCTION

The mechanism by which an individual develops into a male or a female has interested scientists since the time of Aristotle (384-322 B.C.) who wrote of sex determination in sheep and goat in the Historia Animalium "... and if they submit to the male when north winds are blowing, they are apt to bear males; if when south winds are blowing, females." (Thompson, 1910). Unfortunately, these descriptions do not show Aristotle at his best. Little was known about sex determination and differentiation until the end of the last century when improved technology enabled rapid advances to be made.

The field of sexual differentiation has been extensively reviewed by Jost (1958, 1961, 1965, 1970), Burns (1961), Price and Ortiz (1965), Price (1972), and Neumann, Elger and Steinbeck (1970). It is now well known that sexual differentiation involves several steps. The genetic sex of an individual is determined at the time of fertilization.

Normally, the genetic sex determines the subsequent development of the sex organs, general body features and sexual behaviour. The first sexual primordium to appear is the gonadal anlage, and the sexual differentiation of the gonad constitutes the first phenotypic sexual difference. The gonads play a central role in the subsequent development of the genital tract, external genitalia, secondary sexual character of the body as well as the sexual differentiation of the brain. Although the significance of gonadal sex has been recognised, little is known about the factors that determine and control its development.

1.2 Gonadogenesis

The theories about normal sexual differentiation of the male and the female reproductive tract and external genitalia are reasonably

established but gonadogenesis remains an open question. The embryonic origin of different gonadal components has been a controversial subject. The steps by which germ cells are segregated from the rest of the somatic cells are completely unknown. Equally unknown is the factor which determines the initial differentiation of the gonad along one pathway or the other. There are numerous studies of the differentiation of the gonads from the indifferent stage by light and electron microscopy, e.g. rat (Jost, Vigier, Prepin and Perchellet, 1973), rabbit (Allen, 1904), cow (Gropp and Ohno, 1966), pig (Black and Erickson, 1965; Pelliniemi, 1976) and man (Gillman, 1948; Wagnen and Simpson, 1965). In spite of all these detailed descriptions of gonadogenesis, none of the authors considered the problem of gonadal sex differentiation in terms of the differential behaviour of the germinal and somatic elements in the testis and the ovary.

The triple origin of cell types in the gonads has been well recognised: the gonads consist of germ cells, coelomic epithelium and mesenchymal tissue. The latter two are the somatic elements of the gonads contributing to its structural and endocrine function, while the germ cells give rise to the gametes. The germ cells are a unique cell line in the organism that are capable of undergoing both mitosis and meiosis, whereas somatic cells can only divide mitotically. Another characteristic of the germ cells is that in female mammals, both X chromosomes appear to be essential for oocyte division and maturation while only one X chromosome is functionally active in all female somatic cells (Lyon, 1974) (see also 2.3.2).

Before considering the origin of the germ cells, an answer has to be given to the old problem of whether somatic cells can become germ

cells, and whether primordial germ cells can become somatic cells. It is now well established that germ cells are segregated from the somatic cells early in development. The primordial germ cells of the embryo appear to give rise to the entire germ cell population of the adult. If the germ cells are destroyed, e.g. by busulphan (Merchant, 1975), or fail to survive, as in the W^{j} mice (Mintz, 1956), they will not be replaced and gonads without germ cells result (see review by Franchi, Mandl and Zuckerman, 1962). Although somatic cells cannot become germ cells after germ line segregation, there is evidence to show that germ cells can become somatic cells. The testicular teratocarcinoma cells of germ cell origin have been shown to be capable of becoming somatic cells. About 20-40 teratocarcinoma cells were injected into recipient mouse blastocysts, and were capable of giving rise to mosaic mice with several somatic tissues contributed by the injected neoplastic germ cells (Papaioannou, McBurney, Gardner and Evans, 1975).

1.2.1 Origin of germ cells

Many theories have been propounded but the main debate has been between those who believed, as did Waldeyer (1906), that the germ cells differentiated at a relatively late stage from the coelomic epithelium, and those who held with Goette (1875) and Nussbaum (1880) that the germ cells arose at a much earlier stage before the development of the genital ridges, from undifferentiated cells, possibly even during the cleavage stages. The latter view is supported by morphological and experimental evidence.

In some anuran amphibians, primordial germ cells (PGC) can be distinguished from somatic cells throughout embryogenesis by the

presence of a microscopically identifiable cytoplasmic inclusion known as germ plasm (see review by Bourn[illegible], 1934; Blackler, 1970). Descriptive studies have shown that the germ plasm is localised in the vegetal pole cytoplasm and hence the germ cells are located initially in the presumptive endoderm. Later in embryogenesis, the germ cells are reported to migrate from the endoderm into the genital ridge, there to proliferate and then enter meiosis. Although it has now been shown that when the germ plasm is removed surgically or by UV irradiation, no germ cells are formed (Blackler, 1970; Smith and Williams, 1975), the actual mechanism by which the germ plasm specifies the germ cell line has remained unsolved since the time when Weismann (1885) proposed his theory of continuity of the germ cells. The volume of germ plasm has been measured in Xenopus (Whittington and Dixon, 1975). There is no change through cleavage in the embryo and the germ plasm is segregated in a few cells by the normal process of cleavage. Subsequently, the germ plasm takes up a perinuclear position in the cells which undergo a small number of cloning divisions, and this is contemporaneous with the first sign of differentiation.

In mammals, the germ plasm has never been demonstrated, and the time when the germ cells segregate from the somatic cells is not known. The delimiting of the germ cells from somatic cells has still to take place in the 4 1/2 day mouse embryo since a single ectodermal cell from a $4\frac{1}{2}$ day embryo injected into a host blastocyst can give rise to both germinal and somatic elements in the resulting chimaera (Gardner and Rossant, 1976).

The earliest stage the PGCs can be demonstrated in mammalian embryonic development is in the $7\frac{1}{2}$ day mouse embryo, when they are found in the primitive streak (or the mesoderm) (Ozdzenski, 1967). Later,

the PGCs are found in the endoderm of the yolk sac. This indicated that Witschi's observation of PGCs in the endodermal yolk sac of a 13-somite human embryo is only a secondary observation (Witschi, 1948). However, the controversy as to whether the germ cells arise in the extra-embryonic endoderm or mesoderm would seem to be unimportant if the germ cells are segregated before these layers develop.

Once migration of the germ cells has commenced, they move up the stalk of the yolk sac and enter the embryo caudally near to the allantois (Witschi, 1948). They subsequently pass up the gut mesentry and enter the developing gonadal primordia. The various theories which have been proposed for the mode of migration of PGCs are fully reviewed by Franchi, Mandl and Zuckerman, (1961) and Zuckerman and Baker (1977). The PGCs can migrate by (i) active amoeboid movements, (ii) passive movement by differential growth, (iii) passive movement via the blood stream, or (iv) under the influence of a chemical inductor. PGCs cultured in vitro are capable of amoeboid movement (Blandau, White and Rumery, 1963). Electron microscope studies on the morphology of the PGCs in extragonadal sites have confirmed that they are capable of migrating by active amoeboid movement (Zamboni and Merchant, 1973). The energy required for locomotion was dependent on the close association with somatic cells. On arrival at the genital ridges, the cells lose their amoeboid features and assume a structural organisation much simpler than that of the preceeding stage, indicating a short stage of quiescence which is followed by an active mitotic phase.

1.2.2 Differentiation of the gonadal primordia

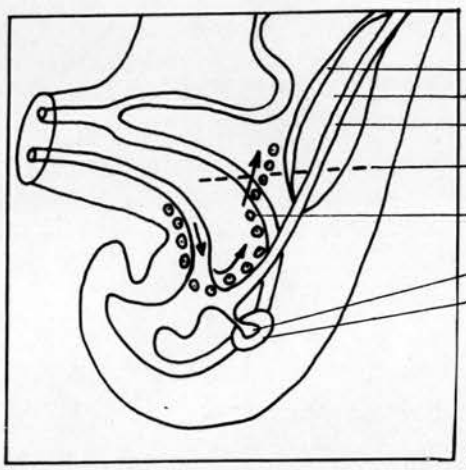
The genital ridges appear much later than the first appearance of the PGCs. The ridges appear in the region of the embryo where the

mesonephros as well as the adrenal cortex differentiate. On each side of the dorsal mesentery, there develops a longitudinal thickening of the coelomic epithelium covering the mesonephros (Fig. 1). This constitutes the genital ridge from which the gonads differentiate. As the mesonephros projects into the peritoneal cavity, it also raises the progressively swelling genital ridge so that the gonad becomes fixed on the internal side of the mesonephros.

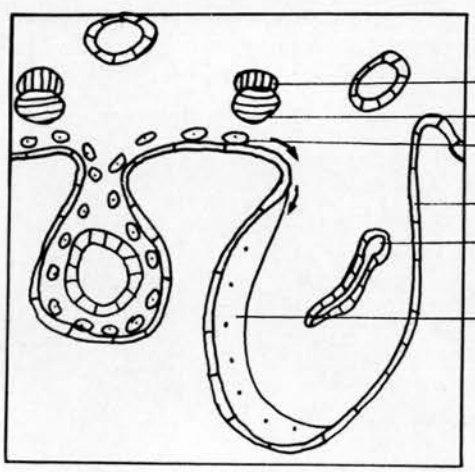
The primordia of the sex ducts also develop in connection with the mesonephros. This relationship between sex organ and the mesonephros is so important that it requires special emphasis. In the adult organism, mature germ cells are discharged from the gonads in opposite directions according to the sex of the individual. In females, oocytes are released outwards from the surface of the ovary into the coelomic cavity before entering the oviduct. By contrast, in the male, the spermatozoa follow an inward direction inside the testis and pass through a complicated system of ducts developed from mesonephric structures before reaching the vas deferens which is derived from the mesonephric duct. These differences are linked to the developmental history of the sex structures.

Detailed study of early gonadal embryology, especially in mammals, is very difficult. Many divergent interpretations or theories have been presented concerning the origin or derivation of the different parts of the gonads (see reviews by Brambell, 1956; Franchi, Mandl and Zuckerman, 1962; Zuckerman and Baker, 1977). In addition, terminology is sometimes misleading because different authors use different terms for the same structure.

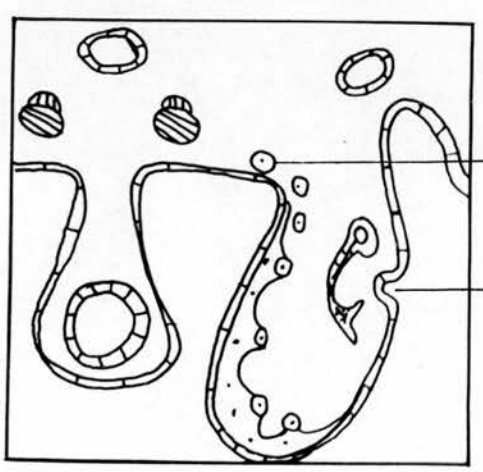
Some classical views of gonadal organogenesis must be recalled. In 1885, Janosik reported that the gonadal coelomic epithelium proliferated as an early set of sex cords into the underlying mesenchyme.



(I) Longitudinal medial section of a five-week human embryo to show the migration of primordial germ cells.



(II) Transverse section showing the gonadal ridge and the migration of germ cells.



(III) Transverse section to show the indifferent gonad.

FIGURE I.A DIAGRAMS TO ILLUSTRATE THE MIGRATION OF PRIMORDIAL GERM CELLS.

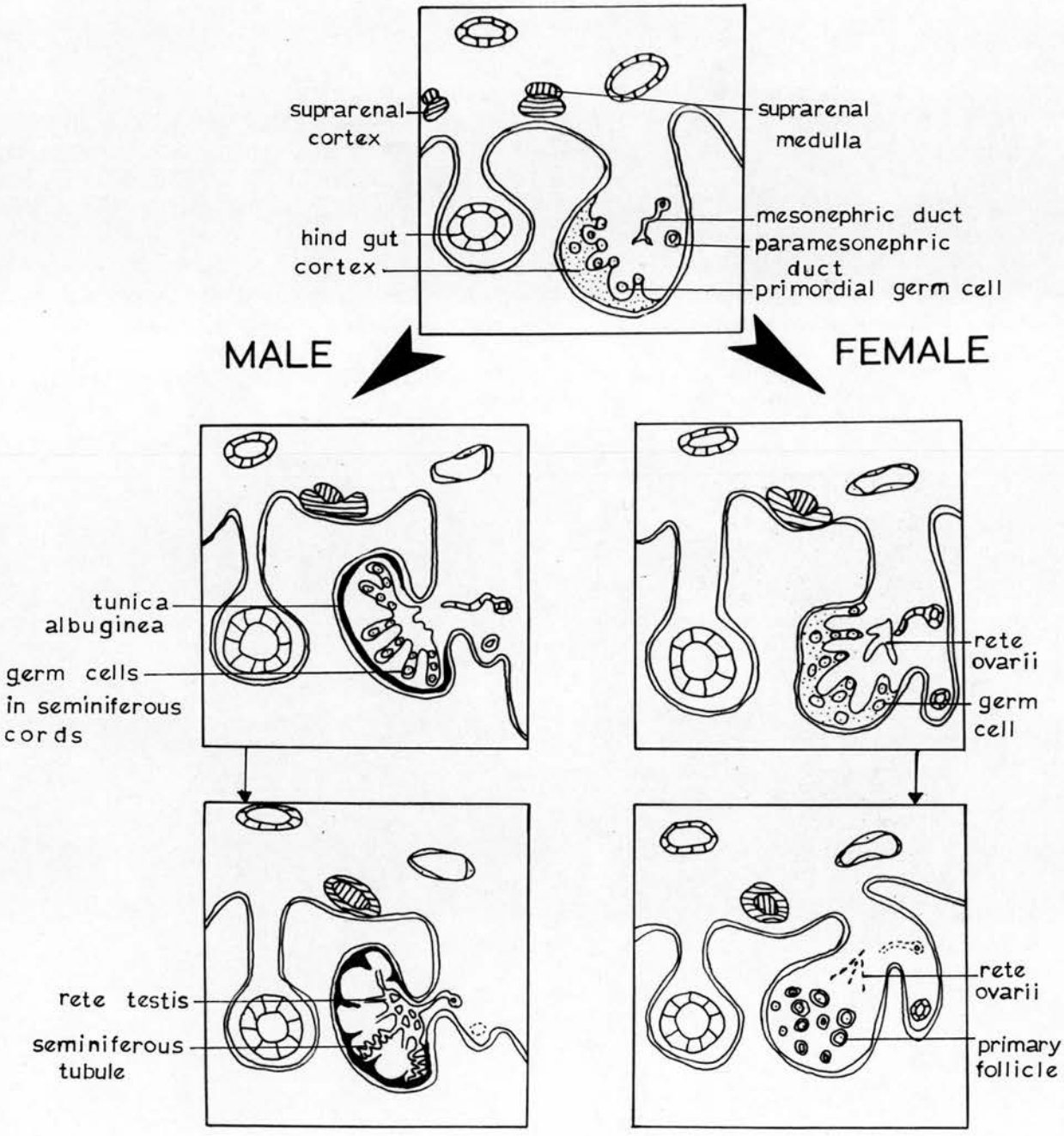


FIGURE 1.B SCHEMATIC SECTIONS TO ILLUSTRATE THE DIFFERENTIATION OF THE INDIFFERENT GONADS INTO TESTES OR OVARIES.

Thereafter, when gonadal sex became histologically discernible, these first sex cords or medullary cords (de Winiwater, 1910) differentiated into seminiferous tubules in males; they usually remained less developed in females. Conversely, the coelomic epithelium flattens over the developing tunica albuginea in the testis, while in ovaries, it remains thickened. Later the persistent coelomic epithelium (sometimes erroneously called germinal epithelium) of the ovary proliferates a second set of cords or Pflüger's cords, which constitute the ovarian cortex. According to this theory, two successive proliferations of sex cords arise from the coelomic epithelium, the first of which has a testicular and the second an ovarian potential. Such a theoretical view must be considered with caution: in many species of animals, sex cords can hardly be recognised in the indifferent gonadal primordia, and cords first become evident when a testis differentiates. The term medulla is equally confusing. Some authors use it when referring to the medullary cords. According to Witschi, the medulla originates from the mesonephros and contributes the rete, the wall of the seminiferous tubules and possibly the interstitial cells.

By looking at a section of an indifferent gonad, the following components can be distinguished: (i) the thickened coelomic epithelium; (ii) a cellular region under the coelomic epithelium, which is considered as cords lying at right angles to the coelomic epithelium; (iii) loose mesenchyme deep in the mesonephros; (iv) germ cells generally beneath the coelomic epithelium.

Several interpretations have been given of the events which permit the formation of the undifferentiated gonadal primordia. According to Gillman (1948), the primary sex cords grow in from the coelomic epithelium. These sex cords interdigitate with the cell layer

in the mesenchyme, making discrimination of the two layers difficult. The germ cells first lie between the growing sex cords; the buds of these cords remain deprived of germ cells and constitute the rete material and the straight collecting tubules.

Fischel (1930) envisaged the origin of the sex cords of the undifferentiated gonad in the cellular region of the mesenchyme beneath the coelomic epithelium. Two types of cells become distinct: polygonal cells that form the sex cords and elongated mesenchymal cells lying between the developing sex cords. Fischel negates the participation of the 'germinal' epithelium in the formation of the ovarian cortex.

According to Witschi (1951, 1956), the coelomic epithelium constitutes the early cortex while the cellular condensation and cords in the mesenchyme form the mesonephric blastema and constitute the medulla of the gonad. A continued condensation of the blastema cells in the innermost region of the gonad finally establishes solid connections between the medulla and the mesonephric tubules, the primordial efferent ductuli. Witschi emphasised the presence of a narrow layer of connective tissue between the cortex and the medulla, ruling out the coelomic epithelium as a source for the medulla. The primordial germ cells are first located in or beneath the coelomic epithelium. The cortex thickens by multiplication of its somatic or germinal cells. At later stages of development, cords are produced bringing germ cells as well as accompanying somatic cells from the coelomic epithelium in contact with the medulla (mesonephric blastema) (Witschi, 1948). Medullary sex cords are thus produced, when in contact with other medullary derivatives.

It is difficult to prove or disprove the different theories because of the varying observations in different animal species. The

picture is less confusing once the PGCs have colonised the gonadal primordia, and the process of sexual differentiation starts to take place. The development of the testis and the ovary has been studied at light and electron microscopic levels in man (Witschi, 1948; McKay, Adams, Hertig and Danziger, 1953; Pinkerton, McKay, Adams and Hertig, 1961; Wartenberg, Holstein and Vossmeyer, 1971; Pelliniemi and Niemi, 1969; Holstein, Wartenberg and Vossmeyer, 1971), monkey (van Wagenen and Simpson, 1965), pig (Allen, 1904; Whithead, 1904; Gruenwald, 1934, 1942; Black and Erickson, 1965; Moon, Hardy and Raeside, 1973; Pelliniemi, 1976), rabbit (Allen, 1904; Gosdon and Conner, 1973; Bjerregaard, Bro-Rasmussen and Reumert, 1974), guinea pig (Black and Christensen, 1969), hamster (Gondos, Paup, Ross and Gorski, 1974; Challoner, 1974), rat (Jost, Prepin and Perchellet, 1973) and in the mouse (Odor and Blandau, 1969a,b; Peters, 1970). A general account of the differentiation of the testis and the ovary is given in the next section.

1.2.3 Differentiation of the testis

Testes differentiate early and rapidly; in female fetuses of the same age, the gonadal anlagen remain histologically undifferentiated although they continue to grow. Histologically, presumptive ovaries are recognised only because they are not testes (Gillman, 1948).

The differentiation into a testis first becomes evident in the human embryo at a crown-rump length of 14 to 16 mm. In the rat, this occurs at Day 14 post coitum (p.c.), and in the hamster, at Day 12 p.c..

The differentiation of a testis is marked by the formation of the layer of connective tissue (tunica albuginea) separating the sex cords from the coelomic epithelium. The sex cords, the future seminiferous tubules, are made up of germ cells and somatic cells (the future Sertoli cells). The cords are separated by smaller and denser intertubular cells. The organogenetic process involved in the formation of the early seminiferous tubules is essentially cellular segregation and association. The germ cells move to the periphery of the Sertoli cells, apparently aided by the cytoplasmic projections in the germ cells (Gondos and Hobel, 1973). In this position, the germ cells become the pre-spermatogonia which are generally arranged in pairs. Pairing is the result of incomplete mitotic division, bridges being retained between daughter cells.

A second important step in testicular development is the differentiation of the interstitial Leydig cells. These cells have been well characterised by electron microscopy, and they appeared in human embryos at the 9th week of gestation, between the intertubular mesenchymal cells (Pelliniemi and Niemi, 1969). In the rat, these cells appear on day 16p.c. (Jost et al., 1973) while in hamsters on Day 14 (Gondos et al., 1974). The origin of the interstitial cells has been attributed to the mesenchyme (Gillman, 1948). According to Gillman (1948) and EM studies (Pelliniemi and Niemi, 1969), the interstitial cells are first represented by spindle-shaped cells but later rapidly increase in size. Biochemical studies have shown that fetal testes are capable of synthesising testosterone (Bloch, 1967). Since the appearance of Leydig cells is followed by the differentiation of the male reproductive tract, it appears likely that secretions of

fetal Leydig cells control differentiation of the genital tract (Zaaijer and Price, 1971; Abramovich, Baker and Neal, 1974). A possibility also exists that hormones produced by the fetal testis may be responsible for the sexual maturation of the central nervous system (Barraclough, 1966). There is no further change in the fetal testis except for the enlargment of the seminiferous tubules and vascularisation.

1.2.4 Differentiation of the ovary

When the undifferentiated gonad does not become a testis, under normal developmental circumstances, it will later become an ovary. The germ cells and the cortical cell layers continue to divide mitotically without showing clear-cut morphological changes. In the centre of the gonad, the rete ovarii (the homologue of the rete testis) becomes recognisable (see section 1.5.1). Superficially, there is no connective tissue layer; the cortical cells remain in continuity with the coelomic epithelium, while in the inner layer of the cortex clumps of cells form into cords which push into the mesenchyme. They are usually referred to as the medullary cords (homologous with the testicular tubules (Forbes, 1942). In the outer cortex, the cords are attached to the coelomic epithelium forming a second set of cords (or Pflüger's cords). This view is supported by Gillman (1948) and Witschi (1948) although Fischel (1930) and Gropp and Ohno (1966) deny any participation of the coelomic epithelium in the development of the gonadal cortex.

An important event takes place in the presumtive ovary. This occurs in human embryos around the 56th day (c.f. Day 14·5 in rats

and on Day 15 in hamsters): the germ cells stop dividing mitotically and they enter meiotic prophase (for the process of meiosis and the control and initiation of meiosis, see section 1.5.3). In human embryos, oocytes in meiotic prophase first appear in the ovary nearest to the rete region. Later in development, oogonia co-exist with oocytes at all stages of meiotic prophase because the process of oogenesis is not synchronised in the human (Baker, 1963). However, in the rat (Beaumont and Mandl, 1962) and hamster (Challoner, 1974), synchrony of female germ cell development is the rule and some 90% of the cells at any particular age are at the same stage of development. The early onset of meiosis in female germ cells is a unique feature that distinguishes them from the male germ cells, which remain undifferentiated until puberty. Another difference between oocytes and spermatocytes is that the meiotic division in oocytes stops at the diplotene stage, and will not resume until shortly before ovulation, while in the male germ cells, meiosis is delayed until puberty, and the division is uninterrupted once started.

Another important event in ovarian differentiation is the growth of the ovary, resulting from the increase in size of the oocytes and proliferation of the somatic cells. This rapid growth is not found in the testis. As a result of the somatic cell proliferation, the sex cords are broken up and eventually, oocytes at the diplotene stage of meiotic prophase become individually surrounded by a layer of pre-granulosa cells (or follicular cells). The primordial follicles first appear in the inner border of the still undifferentiated gonad and progressively the change reaches the surface of the ovary. The primordial follicles then become separated by the ovarian stroma which seems to grow towards the surface from the centre of the gonad.

The origin of the follicular cells is a matter of debate. In a study of ovarian development in the mouse (Byskov and Lintern-Moore, 1973), cat, mink and ferret (Byskov, 1975), the authors maintained that the follicular cells originate from the rete ovarii. Deansley (1972, 1975) studied the development of the fetal ovary in the guinea pig and rabbit and found that there was no correlation between the development of the oocytes and the rete ovarii, but rather that the pregranulosa cells were derived from the sex cords, somatic cells which have a coelomic epithelial origin. The study of the origin of the follicular cells remains to be studied experimentally. Whatever the origin of the follicular cells may be, this remains a critical step in ovarian development because the oocytes which are not surrounded by a layer of pregranulosa cells degenerate. In a study of the calf fetus, Ohno and Smith (1964) suggested that encapsulation of the germ cells with granulosa cells stopped meiotic prophase at the diplotene stage, and was essential for the survival of the germ cells.

In the majority of mammalian species, any surviving pre-meiotic 'stem cells' are eliminated shortly after birth. Hence, the number of germ cells in an ovary is fixed during fetal development and subsequently decreases through atresia and ovulation. However, there is an exceptional group of prosimian primates (lemurs, lorises, the potto and the ayeaye) which have "nests" of primordial germ cells persisting in the ovary throughout adult life. In the loris, the number of cells within the "nests" fluctuates with the phases of the reproductive cycle (Anand Kumar, 1968), being maximal at oestrus and during pregnancy and minimal during anoestrous and lactation. These germ cells can enter meiotic prophase and proceed up to the diplotene

stage but it is unlikely that they contribute to the definitive population of germ cells (David, Anand Kumar and Baker, 1974).

The morphological changes summarised so far afford a basis for the understanding of normal or abnormal gonadal development. However, they give no clue about the mechanisms involved or their causes.

Important data have been gathered on the genetic control of sexual differentiation and the experimental study of normal and abnormal development. Three aspects will be considered: The genetic control mechanisms, experimental studies of normal and abnormal development, and the role of the various tissues that participate in and control gonadogenesis.

1.3 GENETICS OF SEXUAL DIFFERENTIATION

Since the discovery of the sex chromosomes in insects (McClung, 1912), our knowledge of the chromosomal mechanism for sexual differentiation has developed slowly with technical advances in cytogenetics, histochemical staining methods and biochemical analysis. It is known that in man and other mammals, the male is the heterogametic sex (XY) while the female is homogametic (XX), but in birds and Lepidoptera, the reverse is true (the female heterogamety is referred to as ZW and the male ZZ) (Mittwoch, 1973). In mammals, the sex chromosomes have undergone many evolutionary changes. At the cytological level, this is seen in the marked asymmetry of the sex chromosomes: the Y chromosome is considerably smaller than the X, which means that it contains less chromosomal material. At least one X chromosome is necessary for the survival of the cell, and the YY constitution which can be viable in fish (Yamamoto, 1975) is unknown in mammals.

The sex differences can be separated into two components, the presence or absence of the Y chromosome and the presence of two X chromosomes.

1.3.1 The Y chromosome

The X-dosage and the Y-dosage effects in sexual differentiation can be best distinguished in clinical cases of sex chromosome aneuploidy (Burgoyne, 1976): no matter how many X chromosomes are present in aneuploid patients, the Y chromosome is normally essential for male sexual phenotypic development (Table 1). This leads to the

Table 1: Human sex chromosome aneuploids

Karyotype	Genotype	Phenotype	No. of Barr Bodies
Normal	46,XX	F	1
Normal	46,XY	M	0
Turner's Syndrome	45,XO	F	0
Trisomy	47,XXX	F	2
Trisomy	47,XXY	M	1
Trisomy	47,XYY	M	0
Tetrasomy	48,XXXX	F	3
Tetrasomy	48,XXXY	M	2
Tetrasomy	48,XXYY	M	1
Tetrasomy	48,XYYY	M	0
Pentasomy	49,XXXXX	F	4
Pentasomy	49,XXXXY	M	3
Pentasomy	49,XXXYY	M	2

F = Female M = Male

conclusion that the Y chromosome imposes maleness on a system that will otherwise develop into a female.

The Y chromosome might be expected to carry a number of genes, each being concerned with a particular aspect of male development. In man, a region in the short arm of the Y chromosome was thought to be responsible for the differentiation of the testis (Jacobs and Ross, 1966). It is also known, at least in the mouse, that testicular size is also controlled by a Y-linked gene (Hayward and Shire, 1973). However, the key question of how the Y chromosome induces testicular differentiation is still not solved (see section 1.3.3).

1.3.2 The X chromosome

In female mammals, one of the X chromosomes in each somatic cell become genetically inactivated in early development, and the selection of this X is at random in each cell (Theory of X dosage compensation, Lyon, 1961). According to this theory, in aneuploid individuals, all the X chromosomes in excess of one are inactivated. This is in fact demonstrated by the number of Barr bodies, the inactivated heterochromatic bodies demonstrable in the nuclei of cells (Table 1). Therefore, a varying number of X chromosomes does not affect the functional X dosage in the somatic cells.

The situation is different in the germ cells, where both X chromosomes are active in the XX oocytes of mice during their growth phase (Gartler, Andina and Gant, 1975). This is determined by the biochemical measurement of the enzymes produced by the oocytes compared to those produced by the somatic cells. However, XO mice are fertile, but they have a shorter reproductive life than

normal XX mice and the embryos from XO mothers have been shown to develop very poorly in culture when compared with those from their XX sisters (Burgoyne and Biggers, 1976). This effect is thought to be caused by the deficiencies arising during the growth phase of the XO oocytes.

The ability of both the male and the female to undergo masculine differentiation of body sex in the presence of androgen indicates that the regulation of androgen receptors is Y-independent. When the X-linked testicular feminisation (Tfm) mutant strain of mice was discovered (Lyon and Hawkes, 1970), this X-linked gene was found to be responsible for the regulation of the nuclear-cytosol androgen receptor. Hence, the X chromosome is responsible for phenotypic body sex differentiation.

Does the X chromosome take any part in the control of gonadal sex differentiation? The answer to this question was not known until the unusual situation in the wood lemming (Myopus schisticolor) was reported (Fredga, Gropp, Winking and Frank, 1976). In the wood lemming, the male has the normal XY karyotype, but three types of females are distinguished (XX, X$\underline{X}$ and $\underline{X}$Y) by special chromosomal staining techniques (Fredga, personal communication). The result indicates that some female animals appeared to carry a gene on the X chromosome ($\underline{X}$) that can suppress the action of the Y-linked testis organising gene, thus giving rise to $\underline{X}$Y fertile females which produce daughters only.

The wood lemming seems to lend support to Boczkowski's (1971) hypothesis of sexual differentiation in which he postulated that (i) the mammalian gonad will differentiate into a testis unless it is inhibited from doing so, (ii) the specific inhibiting factor is

produced by a structural gene, probably located on the X chromosome, and (iii) the sex determining gene on the X chromosome is rendered inactive by a repressor substance produced by a structural gene located on the Y chromosome.

1.3.3 H-Y antigen and sex differentiation

A gene product of the Y chromosome has not been identified until recently. It now seems as if the histocompatibility - Y antigen could be the product of the testis-determining gene (Wachtel, Ohno, Koo and Boyse, 1975). Rejection of male skin grafts by females from a highly inbred strain of mice is due to H-Y antigen activity (Gasser and Silver, 1972). Recently, a serological method has been developed to measure H-Y antigen activity. Phylogenetical studies showed that H-Y antigen can be detected in all heterogametic animals, i.e. XY male mammals and ZW female amphibians and birds. This H-Y antigen has also been demonstrated in XX human males and XX, Sxr (sex reversed) mouse testicular tissue (Wachtel et al., 1975). Basing on these findings, Ohno (1976) postulated a theory for sexual differentiation: the minimal requirement for sexual development in mammals is the presence of two regulatory genes. The Y-linked gene is responsible for the testicular differentiation, and the testis organised by this gene serves as the source of fetal androgen. The subsequent phenotypic sexual differentiation is dependent on this androgen, as well as the dominant form of the X-linked Tfm gene which regulates the production of androgen receptors without which the cells cannot respond to the hormone. A lot of research still has to be done to establish whether the production of H-Y antigen is

the cause of testicular differentiation, and not a product of the testis.

1.3.4 Apparent autosomal control of sexual differentiation

The control of gonadal sex differentiation is not necessarily limited to the sex chromosomes. There are naturally occurring cases of XX males in man, pig, goat and mouse. These may be caused by autosomal genes exerting their influence on the differentiation of the gonads. In the goat, sex reversal of the XX female is closely associated with a dominant autosomal gene for the lack of horns (Polled). The females (XX) homozygous for this autosomal polled gene become sex reversed and a proportion of homozygous males also become sterile because of congenital aplasia of the head of the epididymis. One obvious explanation for this condition would be the translocation of a portion of the male-determining gene on the Y chromosome to the X chromosome (Ferguson-Smith, 1966). However, careful genetical studies of XX mice and goats fail to support this hypothesis (Ricordeau and Lauvergne, 1967; Cattanach, 1971). What complicates the picture more is that the Sxr gene in the mouse behaves as an autosomal dominant while that in the goat behaves as an autosomal recessive. XX mice with a single dose of Sxr develop as males with small testes which contain germ cells only during fetal and neonatal life. However, sperm were found in the testes of adult XO, Sxr mice, although they were abnormal.

In view of the observations on XX males in the goat, pig, mouse and man, the concept of gonadal sex determination was revised by Hamerton (1968), who proposed that the mammalian X chromosome

carried a male-determining gene, while Y chromosome carried a controlling centre which normally activated this gene to produce a male inductor substance which in turn stimulated the medullary component of the gonad to develop into a testis. In the XX male, an autosomal gene mimicked the normal switching action of the Y chromosome.

1.3.5 Theory of differential growth

A comparative study of the sex chromosomes in vertebrates shows that they have become progressively more powerful in controlling the process of gonadal differentiation which was probably under environmental control originally. Mittwoch (1967) put forward the idea that sexual differentiation is not under the control of a structural gene, but of chromosomal regions that affect the rate of growth and development of the bipotential gonad at a critical stage in development. She discussed the evolution of the male heterogamety system in mammals and pointed out that in placental mammals, embryos of both sexes are in an environment rich in oestrogen; to develop a male phenotype, an embryo must counter this by secreting sufficient amounts of androgen at an early stage of development. The function of the Y-chromosome would therefore be to induce early and rapid testicular development thus enabling it to secrete male sex hormones before the embryo became feminised. By contrast, the avian embryo is not faced with this predicament, and so sex determination with female heterogamety and initial ovarian development is possible.

Mittwoch also studied the embryonic testis of the XX, Sxr mouse. Although the fetal testes of XX Sxr mice are histologically

indistinguishable from those of their XY litter mates (but there is no spermatogenesis in adult XX, Sxr), measurements from serial sections have shown that on Day 15 and 16 p.c., the XX testes are smaller than those from XY embryos (Mittwoch and Buehr, 1973); this could be explained by the lack of a Y chromosome. The lateral asymmetry of the gonads in the chicken, mouse, calf and man is also described as "one of the pointers relating gonadal differentiation with differential rates of growth" (Mittwoch, 1975). The chief criticism of this theory is whether the difference in growth rate is the cause or the consequence of sexual differentiation.

1.4 EXPERIMENTAL STUDIES OF GONADAL SEX DIFFERENTIATION

Whilst embryological studies of normal gonads have contributed to our understanding of gonadogenesis, a tremendous amount of information on the mechanisms of sexual determination and differentiation has emerged from experimental work in mammals and other vertebrates. Although gonadal sex reversal has been achieved experimentally in fish, birds, reptiles and amphibians, experiments in mammals have so far been unsuccessful, with the exception of those reported by Burns (see review, Burns, 1961) who claimed to have reversed the gonadal sex of opossums. Recently, similar success has been reported in wallaby (Macropus eugenii) (Alcorn, 1975). The natural experiment of the freemartin, a female intersex cow born cotwin to a male (Tandler and Keller, 1911) was the pioneer work in this field.

1.4.1 Freemartins and the hormonal theory

Tandler and Keller (1911) were the first to appreciate the occurrence and significance of an anastomosis between the blood vessels of the fused chorions of heterosexual bovine twins, and they correlated it with the hypogenital condition of the female (a freemartin). No theory was proposed for the causation of the freemartin condition until in 1917, when Lillie put forward the hormonal concept. In this theory, the masculinization of the gonads, reproductive ducts and accessory glands of the freemartin were attributed to male sex hormones from the testes of the male co-twin. This concept was based on a thorough examination of the vascular

anastomosis between the chorions, and anatomical and histological studies on the freemartins, their co-twins, and normal controls.

The publication of the hormonal theory aroused interest in the field of sex differentiation; various experiments were tried in mammals in attempts to produce freemartin gonads experimentally by surgery, in vitro culture, and drug injection. In summary, the most important findings were that the initial differentiation of the gonads was independent of hormones; Jost (1947) showed that gonadogenesis proceeded normally in decapitated rabbit fetuses, and excised indifferent gonads differentiated when maintained in culture (Wolff and Haffen, 1965). The administration of steroid sex hormones to fetuses did not affect gonadal differentiation in eutherian mammals, but androgens produced various degrees of masculinisation of the female reproductive tract (Neumann, Elger and Steinbeck, 1970) and experiments with anti-androgens, notably cyproterone acetate, confirmed this (Jost, 1972/3; Neumann, Elger, Steinbeck and Gräf, 1975). Gonadectomy in utero (Jost, 1961) and culture of reproductive tracts in vitro (Price, 1972, 1975) showed the importance of testicular hormones in normal sex differentiation in the male, and the hormone independence of the female. The dual secretory activity of the testis was well documentated, androgens stabilizing the Wolffian ducts (differentiation of epididymides, ductuli deferentia, seminal vesicles), differentiation of the accessory sexual glands derived from the urogenital sinus (prostate, coagulating glands, bulbourethral glands) and the differentiation of male external genitalia (penis, scrotum). Furthermore the Mullerian inhibitory factor, apparently secreted from the Sertoli cells of the testis

(Blanchard and Josso, 1974; Josso, 1975) was responsible for the regression of the Mullerian duct and possibly the descent of the testes.

In the light of recent research, the hormonal theory appears to apply to the differentiation of the reproductive tract, and accessory glands, but not to the gonads. However, the bovine placenta has been found to be active in metabolising androstenedione and testosterone to oestrogen, indicating that androgens from the male co-twin are unlikely to reach the freemartin (Pierrepoint, Stewart and Rack, 1969). Short, Smith, Mann, Evans, Dickson, Freyer and Hamerton (1969) found that the freemartin gonad secreted large amounts of testosterone, so that the freemartin could be masculinized by the secretion of its own gonad. But in vitro studies on post-natal freemartin gonads showed no correlation between the androgen production and the degree of masculinization of the sex ducts (Pierrepoint et al. 1969). In view of all this evidence, the hormonal theory no longer seems tenable, and the cause of gonadal sex reversal in the freemartin remains unknown. Witschi's cortico-medullary theory could perhaps be invoked to explain the freemartin gonads.

1.4.2 The cortico-medullary antagonism theory

Another theory of sexual differentiation, possibly applicable to mammalian gonads, was proposed by Witschi (1970) from his research on gonadal development in amphibians. In 1914 and 1915, Witschi studied sexual differentiation in frogs, and began the development of his theory of the cortico-medullary inductor system in

gonadogenesis. He found that the undifferentiated gonad of the frog was potentially bisexual, consisting of an outer cortical and an inner medullary region. His theory (Witschi, 1957) proposed that the development of the testis from the medullary region was a result of the dual action of an inductor, 'medullarin', and an inhibitor, 'anti-corticin', which suppressed the development of the female cortex; ovarian development, on the other hand, is the result of the action of the inductor 'corticin' and the inhibitor 'anti-medullarin'. He projected his theory to mammals and offered an interpretation of the freemartin - a natural experiment in parabiosis (Witschi, 1965). Shortly before Witschi's death, he restated his theory (Witschi, 1970) that corticin, presumably elaborated by the gonadal cortex, was a weak inductor which had the capacity only in the absence of medullarin to stimulate the medullary part of the undifferentiated gonad to become ovarian tissue; medullarin was described as a powerful inductor of the gonadal medulla which transforms it into testicular tissue. Although this theory received wide support, it is still open to doubt because none of these inductors and inhibitors has yet been identified.

The latest study of the bovine freemartin showed that if the vascular anastomosis between the male and the female fetuses was ligatured in early gestation, the inhibition of gonadal differentiation and Müllerian duct development in the female could be prevented, although the female fetuses were found to be XX/XY chimaeric in the liver tissue (Vigier, Locatelli, Prepin, du Mesouil du Brisson, and Jost, 1976). This conclusively showed that a diffusible factor rather than the XY cells from the male co-twin must be the cause of

of the freemartin condition. The factor requires identification, but it may well be the 'medullarin' proposed by Witschi.

1.4.3 In vivo studies of gonadal sex reversal

In contrast to the result obtained with fish, birds and amphibia, the administration of sex steroids to mammalian embryos on the whole has been unsuccessful in affecting the gonadal sex of the embryo (see Burns, 1961; Wolff, 1962). The effect of extraneous sex hormones on early embryos is often lethal, and if they are administered later, the time at which sex reversal is possible may have passed. The only clear-cut results on the effects of sex hormones on gonadal differentiation of a mammal have been obtained by Burns (1950, 1956) in a marsupial. He reported that the testes of the opossum (Didelphys virginiana) could be induced to differentiate into ovotestes or ovaries through the injection of minute amounts of oestradiol dipropionate to the ambisexual young residing within the marsupium. This is the only reported instance of gonadal sex reversal among mammals through the use of steroid hormones, and involves converting a testis to an ovary. Similarly, Alcorn (1975) found that the low dosage oestrogen treatment of tammar (Macropus eugenii) male pouch young produced testicular modifications consistent with those reported by Burns (1956).

Among the eutherian mammals, sex steroid injections and gonadal grafts can induce important modifications of the genital tract, although complete sexual reversal of the gonads has never been achieved. However, some inhibition and intersexuality of the gonads has been obtained by grafting in parabiosis.

MacIntyre (1956) transplanted pairs of ovaries and testes from Day 16 p.c. rat fetuses beneath the kidney capsule of adult rats castrated three weeks before the operation. The grafts remained in place for three weeks, after which ovarian differentiation was suppressed in some explants, and the grafts showed ovarian rudiments with degenerated follicles. The development of follicles was inhibited in most cases. He also showed that ovarian tissue could be transformed into testicular tissue; in seven cases out of 23, he observed seminiferous tubules containing oocytes. Holyoke (1957) obtained similar results with 17-24 day rabbit fetuses. Similarly, Turner and Asakawa (1964) took testes and ovaries from $12\frac{1}{2}$ to $14\frac{1}{2}$ day fetal mice and grafted them beneath the renal capsule of castrated adult male mice. They showed that in ovaries that had been masculinized by the adjacent testes, the oogonia could develop into spermatocytes.

In another experiment, MacIntyre, Baker and Wykoff (1959) were able to show suppression of testicular differentiation resulting from some transplants of gonads of various age combinations in which the ovary was older than the testis. The greatest percentage (36%) of cases showing testicular suppression was achieved when the ovary was 16 days (p.c.) old and the testis, 13 days. The authors concluded that it had been possible to demonstrate the existence of a substance secreted by the developing mammalian cortex and capable of suppressing the differentiation of the gonadal medulla. The authors felt that their evidence, combined with that from the previous study (MacIntyre, 1956), indicated that Witschi's theory of the cortico-medullary induction of gonadal differentiation applied to placental mammals.

In a separate experiment, MacIntyre, Hunter and Morgan (1960) showed that the ovary and testis had to fuse intimately to obtain an inhibitory effect. They grafted the testis and ovary at varying distances apart from one another under the kidney capsule (1 to 10 mm) and showed that when the two gonads were more than 8 mm apart, the testis no longer affected ovarian development. The results suggested that the chemical mediator of testicular differentiation in the rat was localized in its effectiveness.

Recently, transplantation experiments indicated that the sex reversal of the ovary may not be mediated by a chemical mediator secreted by the testis. Mangoushi (1975) transplanted fetal rat ovaries into the scrotal sacs of castrated or intact adult rats and found that all the ovaries in the scrotal sac were inhibited in development and in some cases, tubular structures similar to seminiferous tubules developed, while all the ovaries transplanted under the kidney capsule differentiated normally. The author proposed that it was the lower temperature in the scrotum that caused the change.

1.4.4 In vitro studies of gonadal sex reversal

When gonadal explants are grafted into the body of a host, and become well vascularised, there are still problems principally related to the histocompatibility status of graft and host tissues. Time also has to be allowed for the host-graft relationship to become established. When explants are cultured *in vitro* there are no histocompatibility problems and the culture conditions can be varied at will. However, the explants are in a poorer state of

nutrition and respiration, and once they have attained a certain size, their further survival is severely handicapped. In vitro cultures of undifferentiated mammalian gonads can undergo normal sexual differentiation; if the gonads are explanted after sexual differentiation, the ovaries and testes continue to differentiate up to the stage of gametogenesis (Haffen, 1970).

Organ culture of male and female gonads has also been carried out. Holyoke and Breber (1958) cultured heterosexual fetal rabbit and rat gonads. In all cases, the development of the ovaries was retarded and they did not develop cortical elements. In three cases, structures resembling testicular cords were also observed in the ovarian medulla.

When a mouse ovary was cultured with a chicken's testis (Salzgeber, 1960), the two organs fused intimately. When collected at 17-19 days p.c., the ovaries developed follicles, but follicular cells were lacking in some cases and flattened in others. The changes in the ovaries were more marked if they were associated with a chicken testis at 13-14 days. The ovaries developed in an atypical fashion consisting of loose mesenchyme, and cortical and medullary zones could not be distinguished. A few isolated oocytes were able to persist in the undifferentiated tissue, and tubules of the rete ovarii could be clearly distinguished, appearing as sinuous tubules with walls composed of a layer of irregularly arranged cells, penetrating into the chicken testis to form a mixed structure with the seminiferous tubules. A few cords were occasionally found in the vicinity of the rete which sometimes contain oocytes or degenerating follicles.

In summary, organ culture or transplantation parabiosis of male and female gonads induces both inhibition of differentiation and a degree of intersexuality. The mammalian ovary reacts when it is paired with a testis; ovarian development is generally inhibited, and the medulla shows various degrees of testicular development. In some cases sex reversal of germ cells was seen, with oogonia differentiating into spermatogonia or even spermatocytes in testicular tubules within the ovary.

1.5 THE ROLE OF GONADAL COMPONENTS IN ORGANOGENESIS

Normal gonadogenesis has been shown to be basically determined by the genetical constitution of the gonad, but the normal course of development can be altered experimentally. It is therefore appropriate to consider the role of the diverse tissues that take part in gonadogenesis.

1.5.1 Role of the mesonephros and the rete in gonadogenesis

The role of the mesonephros in gonadogenesis was studied in several submammalian species with varying results. The general theme behind the experiments depended on the fact that the development of the mesonephros from its early blastema was induced by the primitive ureter, which, if prevented from reaching the mesonephric area, caused the nephric structures to fail to differentiate. In the newt, Pleurodeles waltlii, when there was complete mesonephric agenesis, no gonad formed at all (Houillon, 1956); the primordial germ cells only accumulated beneath the coelomic epithelium. In cases where isolated mesonephric islands developed, they furnished the medullary tissue, and rudimentary gonadal nodules differentiated in this area.

In the chick embryo, stopping growth of the Wolffian duct produced mesonephric agenesis, but it did not prevent small ovaries and testes from being formed (Bishop-Calame, 1966). These gonads lacked definite mesonephric connections but they received medullary structures from a primordium common to the adrenal cortex. Similar results have been briefly reported in the frog, Rana dalmatina

(Cambar and Mesnage, 1963). These experiments support the concept that the presence of a medullary component is a prerequisite for the development of the gonad, although it may be difficult to assess from which cells in the mesonephros-adrenal-cortex-medulla blastema it is formed.

There is no evidence available in mammals to indicate the involvement of the mesonephros in early gonadogenesis. Many embryologists considered the rete blastema to be mesonephric in origin (Waldeyer, 1870; Wallart, 1928; Witschi, 1951). In the testis, the rete testis forms the structural part connecting the seminiferous tubules with the vasa deferentia. In the female, the rete ovarii (which is homologous to the rete testis in the male) does not become the reproductive duct system and its function is not well known. The rete ovarii takes up different morphological appearances in different species of mammals. It is quite large and compact in many species e.g. in one genus of bats, Uroderma, it differentiates into a rete type of interstitial gland which resembles an endocrine gland whose exact function is not well known (Mossman and Duke, 1973). In the jumping mouse (Zapus), the guinea pig and the white-tailed deer (Odocoileus virginianus) the rete epithelium is columnar and the lumen wide, indicating an exocrine secretion into the rete. There is recent evidence showing that the rete ovarii produces a factor that initiates and controls meiosis in the mouse ovary (Byskov, 1974) (for details, see section 1.5.3).

There are, however, other embryologists who believe that the rete ovarii does not originate from the mesonephros, but that it develops from the mesenchymal tissue (Wichman, 1916; Sauramo, 1954) or from the surface epithelium (Coert, 1898; Politzer, 1933; Gillman,

1948; Watzka, 1957). In organ cultures of rat ovaries, there is a suggestion that it is the mesonephros rather than the rete ovarii that is essential for the initiation of meiosis (Rivelis, Prepin, Vigier & Jost, 1976). If this question is to be settled, the origin of the rete ovarii will have to be reinvestigated using such techniques as radioactive labelling of cell types.

1.5.2 Role of germ cells in gonadogenesis

From the morphological study of gonadogenesis, germ cells seem to play an inert role. It is generally accepted that the somatic components of the indifferent gonad can develop into a testis or an ovary independent of the presence or absence of the germ cells, at least in the initial stages. The W allele in the mouse severely depletes the number of germ cells, and yet the testis or ovary develops normally (Coulombre and Russel, 1954; Mintz, 1957). Busulphan treatment of the mouse embryo selectively kills the germ cells but does not interfere with the initial stages of gonadal differentiation.

As far as the testis is concerned, endocrine activity can proceed normally in the absence of any germinal tissue. This is illustrated in the XXY males in man and animals who lose the germinal cells at some stage prior to puberty but the androgen secretion of the gonad is not necessarily impaired.

In the ovary, it seems that the endocrine activity of the somatic tissue is entirely dependent on the survival of the germ cells. Light and electron microscope studies on busulphan treated rats showed that the presence of germ cells in the epithelial cords

at birth was essential for the structural differentiation of somatic cells into steroid-synthesising cells in the ovary (Merchant-Larios, 1976).

1.5.3 Meiosis in the germ cells and the control of meiosis

The process of meiosis consists of two cell divisions, each of which consists of four stages: prophase, metaphase, anaphase and telophase. The first meiotic division is a very complex one, extending over a prolonged period of time. The first prophase can be divided up into four subphases: leptotene, zygotene, pachytene and diplotene. The process has been studied in rat, mouse, hamster and guinea pig testis (Leblond and Clermont, 1952), and human testis (Clermont, 1972). In the ovary, the process has been described for many species and reviewed by Baker (1972), White (1973) and Zuckerman and Baker (1977).

Although both male and female germ cells undergo the same process of mitosis, meiosis and maturation, there are however three major differences between them. First, the male germ cells start meiosis at puberty while female germ cells usually start meiosis during fetal life, although a few exceptional mammalian species start meiosis just after birth (see Baker, 1972). Secondly, the process of meiosis in the male germ cells occurs in 'waves' in the seminiferous tubules with typical cell associations and is an uninterrupted process, while in the female it is an 'on-off-on-off-on' process. Thus, as a rule, meiosis in oocytes starts during fetal life but becomes arrested at diplotene stage (so-called 'resting phase'; dictyate or dictyotene) by shortly after birth and will not resume meiosis until shortly before ovulation. The egg

is usually ovulated at second metaphase which is a second resting phase, and meiosis will not be completed until the ovum has been fertilised by a sperm. The third difference between the male and female germ cell is that the division of the oocyte is unequal while in the male it is not. In the first reduction division, a secondary oocyte and the first polar body are formed; subsequently the secondary oocyte gives rise to the definitive gamete (ovum) and the second polar body in the second meiotic division. Unequal division does not occur in spermatocytes.

What controls the initiation of meiosis in the germ cells? Experiments done in Crustacean (Charniaux-Cotton, 1965) and in Molluscan (Streiff, 1967) germ cells indicate that oogenetic evolution including meiosis, occurs as a process of autodifferentiation, while spermatogenic evolution is imposed by a hormone produced by the androgenic gland in Crustacea or by the cerebral ganglion in Mollusca. In mammals, it is difficult to assess whether meiosis is actively prevented in the immature testis or whether it is actively induced in the ovary, or whether both controls operate.

Evidence gathered so far indicates that meiosis in the female germ cells is induced by a meiotic inductor substance. It is well known that ovaries removed from an early embryo cannot be maintained in culture; the germ cells fail to enter meiosis and they subsequently degenerate (mouse: Wolff, 1952; sheep: Mauléon, 1971; hamster: Challoner, 1975a). However, meiosis can proceed in vitro if the ovaries are removed after a critical stage of fetal development (Martinovitch, 1938; Borghese and Venini, 1956; Baker and Neal, 1973). Byskov (1974) transplanted fetal mouse ovaries into

nude mice and showed that the external rete ovarii was necessary to trigger the onset of meiosis in the germ cells. This proposal is also supported by the histological studies of cat, mink and ferret ovaries. Cellular contact between intraovarian rete cords, groups of germ cells and the surface epithelium can be seen and the germ cells in the innermost part of the cortex, in contact with the rete cells, are found to be the first to enter meiotic prophase. A PAS-positive secretion is also found in the lumen of the extra-ovarian rete tubules and is thought to be the meiotic inductor factor secreted by the rete cells. Recently, a French group of scientists (Riveli et al., 1976) cultured fetal rat ovaries with part or whole of the mesonephros which was also found to be essential for the survival and initiation of meiosis in the germ cells. But, they did not exclude the rete from the mesonephros. There are embryologists who believe that the rete derives from the mesonephros (see section 1.5.2) and the argument as to whether it is the rete or the mesonephros that initiates meiosis in the female germ cells would not be important. If the initiation of meiosis in the female germ cells is actively induced by a diffusible factor, the cause of synchronous and asynchrous meiosis observed in different mammalian species requires reconsideration.

In the male, the early envelopment of the germ cells by the seminiferous tubules, and the association of the germ cells inside the tubules could account for the absence of meiosis until puberty. Burgoyne (1977) also suggested that the Y chromosome may play a role in "switching off" the early action of the meiotic inductor factor. Endocrine factors are also thought to be responsible for the initiation of meiosis, although we still know very little about

the control of the male germ cell differentiation. If the female germ cells enter meiosis under the influence of the inductor emanated from the rete ovarii, is there an equivalent substance being secreted by the male homologue, the rete testis? This remains an open question.

1.5.4 Germ-soma interaction

It is not clear whether it is the somatic tissue that determines the differentiation of the germ cells or whether it is the germ cell sex that determines the fate of the gonad. The study of germ cell/somatic cell interactions is important in the study of gonadogenesis and sex differentiation. Up to date, this problem has only been considered by a few authors (for reviews see Short, 1972, Burgoyne, 1977; O and Short, 1977).

The passive role of germ cells in the initial stage of gonadogenesis is well established from embryological studies (see section 1.5.1). The genotype of the germ cells may even be completely different from that of the somatic cells. This is well illustrated by many examples in different mammalian species. In the creeping vole, Microtus oregoni, the male has normal XY somatic cells, but the germ cell line loses the X chromosome, giving OY germ cells; the females on the other hand have an XO soma and XX germ cells which are thought to arise from mitotic non-disjunction of the X chromosome (Ohno, Janichill and Stenius, 1963). The Australian bandicoots (Family Peramelidae) produce normal XX and XY germ cells but the somatic cells of both sexes are XO. The Y chromosome in the male and one of the X chromosomes in the female is lost from somatic cells in early pouch life (Hayman and Martin, 1965). The

most recent example of sex-soma dimorphism is the wood lemming (Myopus schisticolor). Cytogenetic study shows that there are XY females that have XX germ cells, which have presumably arisen through the loss of the Y chromosome and doubling of the X chromosome by mitotic non-disjunction (Fredga et al., 1976).

1.5.5 Natural chimaeras

Sex chromosome mosaicism in man for an aneuploid and a normal cell line of the same gender (e.g. XO/XX) results, as expected, in an amelioration of the defects seen in ~~men~~ human aneuploids, e.g. XO. Mosaicism for cell lines of different gender is of more interest. The most frequently reported cases are XO/XY or XO/XYY, and they exhibit a range of phenotypes from Turner's syndrome, through true hermaphroditism, to fairly normal males. The true hermaphrodites may have an ovotestis or an ovary + ovotestis on one side and a testis or ovotestis on the other (Ferrier and Kelly, 1967), depending on the proportion of the two cell types in the two gonads. This is obviously extremely difficult to assess.

In the freemartin condition in cattle, sheep and goats, XX/XY leucocyte chimaerism is evident. On the basis of this a "Cellular theory" was put forward by Fechheimer, Herschlar and Gilmore (1973) and Stewart (1965) to explain the aetiology of the condition. In brief, their theory states that the freemartin's ovaries and sex ducts are masculinized by the direct action of XY cells from the male cotwin. Ohno, Trujillo, Stenius, Christian and Teplitz (1962) demonstrated mitotic XX cells in the testes of neonatal bulls cotwin to freemartins and raised the possibility that these cells could be primordial germ cells that had crossed over from the freemartin's ovary.

Alkaline phosphatase positive presumptive germ cells, were found in the blood vessels of triplet fetuses at 30 days of gestation, the time when migration of germ cells was in progress (Ohno and Gropp, 1965).

In XX/XY chimaeric bulls, the testes may receive a dose of XX germ cells from the freemartin cotwin. Teplitz, Moon and Basrur (1967) have claimed to have detected XX germ cells, including primary spermatocytes at diakinesis-metaphase, in three XX/XY bull calves aged 2, 10 and 11 months. However, the question of germ cell chimaerism is now very much in doubt. Stone, Berman, Tyler and Irwin (1960) showed no germ cell exchange even between chimaeric individuals of the same sex (the XY/XY chimaeric bulls transmitted red cell antigens to progeny only from one of the two cell lines). Furthermore, testicular preparations obtained from 7 bulls aged 44-56 weeks, born twin to freemartins have been studied by Ford and Evans (1976), and no spermatogonia or spermatocytes with two X chromosomes could be identified. It therefore seems unlikely that XX germ cells contribute to the production of functional spermatozoa although the possibility could not be excluded. This suggests that XX germ cells are unable to survive in a testicular environment.

Similarly, the freemartin gonads may also receive a dose of XY germ cells. However, even if this occurs it appears that they are destroyed during development. A study of the development of fetal freemartin gonads showed that the germ cells ceased to multiply between 50-60 days p.c.. In the normal bovine ovary at this time, a 90-fold increase in germ cell numbers was observed (Jost, Perchellet, Prepin and Vigier, 1975). The germ cells become scarce in the freemartin gonad after 150 days and disappear after the 5th month of

pregnancy. The cause of this disappearance is not yet clear. Short (1971) discussed the problem and attributed this to the inability of the freemartin's own XX germ cells to survive in a testicular environment.

In accord with the recent proposal that the H-Y antigen may be the product of the Y-linked testis determing gene, testicular organisation has always been found to be associated with the expression of the H-Y antigen, even in exceptional individuals whose gonadal sex apparently does not agree with their sex chromosome constitution (Wachtel et al., 1975). Ohno, Wachtel, Koo and Christian (1976) were able to demonstrate the H-Y antigen in freemartin gonads. They discussed the results and revived the Cellular theory of freemartinism, by proposing that the minority of XY cells in the freemartin gonad are able to produce the H-Y antigen and so induce the XX cells to engage in testicular organisation. The idea no longer seems attractive particularly in view of the recent work of Vigier et al. (1976) who demonstrated conclusively that chimaerism itself cannot be the cause of freemartin's gonadal hypoplasia. (see also 1.4.1).

The other well known example of natural chimaerism is the dizygotic twins of marmosets where vascular anastomoses between the twins are common. The freemartin condition has never been observed in the female twin of heterosexual marmoset pregnancies. In a search for germ cell chimaerism, Benirschke and Brownhill (1963) claimed to have found XX spermatogonia or spermatocytes in the testes of three species of marmoset although their chromosomal spreads do not necessarily come from spermatogonia, and could have come from XX leucocytes. Hampton (1970) also claimed to have seen XY oocytes in some females and XX spermatocytes in some males. Ohno (1976)

explained the absence of the freemartin condition in the XX/XY female marmosets on the basis of histocompatibility genes which might interfere with H-Y antigen transfer between the two cell lines.

1.5.6 Experimental chimaeric mice.

Chimaeric mice can be produced experimentally by in vitro aggregation of cleavage stage mouse embryos of different genotypes (Tarkowski, 1961; Mintz, 1962, 1964). It was found that in the XX/XY chimaeric mice, the animals usually develop a male phenotype (McLaren, 1972). No XX germ cells have ever been found among the primary spermatocytes in the male XX/XY chimaeras. (Mystkowska and Tarkowski, 1968). Subsequent studies on the fetal testes of sex chromosome chimaeras showed that cells in meiotic prophase were found between $14\frac{1}{2}$ and $18\frac{1}{2}$ days p.c. (Mystkowska and Tarkowski, 1968; Kofman-Alfaro and Chandley, 1970). Using a tritiated thymidine labelling method, the characteristic late labelling pattern of the Y chromosome was not observed in those meiotic germ cells. The authors concluded that the meiotic cells were likely to be XX rather than XY cells. This line of evidence therefore also showed that XX germ cells cannot survive in the testicular environment.

Can XY germ cells survive in an ovarian environment, responding to the ovarian meiotic inductor factor to form an oocyte? The nearest answer to the above question comes from the study of an exceptional XXY male mouse obtained from a XX/XY chimaeric mother (Ford, Evans, Burtenshaw, Clegg, Tuffrey and Barnes, 1975). It is genetically evident that the Y chromosome of this exceptional male could only have been derived from its chimaeric mother. It is likely

that it resulted from fertilization of an XY egg by an X-bearing sperm. Nondisjunction of the X and Y chromosome during meiotic division of an XY germ cell would produce an XY oogonium which would give rise to XY eggs. Burgoyne and Biggers (1976) predicted that if an XY germ cell completed oogenesis, then, like an XO germ cell, it should suffer from the consequences of X dosage deficiency which would lead to impairment of preimplantation development. Thus the embryos derived from the XY germ cells of XX/XY female chimaeras may not be able to compete effectively with embryos from the XX germ line. Whatever the correct interpretation may be, the finding clearly indicates that the presence of a Y chromosome in an oogonium does not prevent the production of a functional egg. More experiments have to be done to find out whether XX non-disjunction is the rule in XY oogonia.

The main criticism of looking at genotype-environment relationships and the germ-soma relationship in the chimaeric mouse model is that, at present, there is no way of knowing the 'patch size' of the chimaeric cells. New techniques therefore have to be developed to solve the unanswered questions.

CHAPTER II AIM OF THE STUDY

Factors governing sexual differentiation of the gonads are not yet sufficiently elucidated. It is generally assumed that at a certain stage of embryogenesis, some cells are genetically activated or derepressed to impose gonadal organogenesis. However, the gene products to initiate gonadogenesis remain unknown. Equally unknown is the question of whether the differentiation of an early ovary results from genetic activation of some cells producing one or more inductor substances or merely results from the absence of testicular inductor, as suggested by the prolonged undifferentiated condition of the ovary as compared with the testis. Some factors are known to initiate meiosis in the female germ cells; in the bovine freemartin, some factors are also recognised which cause disorganisation and inhibition of ovarian development.

Although all the experimental investigations of mammalian gonadal differentiation indicate that the testis is acting strongly and seems to be imposing its influence on ovarian development, there is no evidence as to whether or not there is an ovarian inductor. This may be due to the fact that testis develops earlier than the ovary and also the technical difficulties in maintaining early isolated ovaries in culture. With improved culture techniques, this problem can now be reinvestigated.

If the theory of cortico-medullary antagonism proposed by Witschi to explain sexual differentiation in amphibians is valid for mammals, it seems entirely possible that embryonic inductors "corticin" and "medullarin" might be effective over short distances, as seemed to be the case in some of the amphibians. A developmental mechanism of this sort could explain the initial differentiation of

an indifferent mammalian gonad into an ovary or a testis, since the male and female primordia (medulla and cortex) are in contact. With these thoughts in mind, the main objective of this Thesis has been to grow embryonic male and female gonads together with one another. This would in effect be placing tissue in which differentiation is chiefly cortical next to tissue in which differentiation is chiefly medullary.

The process of sexual differentiation in the gonads can be divided into the differences in somatic organisation and in the process of meiosis in the germ cells. Although the cause of the initiation of somatic differentiation in the gonads remains unknown, meiosis of the female germ cells in the mouse ovary has been found to be initiated by a factor emanating from the rete ovarii (see section 1.5.3). The initiation of meiosis in the female germ cells can be studied in vitro. By this means, it was also hoped to find out whether the male germ cells responded to the female meiotic inductor.

It is not clear whether it is the somatic tissue that determines the differentiation of the germ cells or whether it is the germ cells that determine the fate of the gonad. The XX/XY chimaeric mouse forms the best model to look at the germ-soma relationship, but there is, at present, no way of knowing the "patch size" of the chimaeric cells. Techniques are now available to separate the germinal and somatic cells from adult rat testis (Steinberger and Steinberger, 1965). In the present study, the method was adapted to separate the germinal and somatic cells in the fetal hamster ovary and testis. The separated XY germinal cells were then mixed with the XX somatic cells, or vice versa, reaggregated and cultured. This

provided another model for looking at the germ-soma relationships and attempting to solve the unanswered questions about the sexual differentiation of the gonads.

In summary, the following experiments were performed:-

(1) using *in vitro* culture techniques, the role of the rete ovarii in the hamster ovarian and testicular development was studied; the nature of secretion from the rete ovarii was also investigated by culturing the rete ovarii, separated by a millipore filter, from the fetal testis;

(2) the development of fetal rat gonads was studied by transplanting them under the kidney capsule of castrated adult rats;

(3) the germinal cells and somatic cells relationship was investigated using the XX(germ)/XY(soma) and XY(germ)/XX(soma) chimaeric gonads.

CHAPTER III MATERIAL AND METHODS

3.1 Animals

3.1.1 Hamsters

Golden hamsters of the species Mesocricetus auratus (Waterhouse) were obtained from an accredited hamster breeder (G. Gore, Laidon, Essex, U.K.). They were maintained in conditions of controlled daylength (14 hours daylight and 10 hours dark), and fed Oxoid breeding diet and water ad libitum. The stage of the oestrous cycle was determined externally from the vaginal discharge following the method of Ward (1948). Adult hamsters were mated on the evening of pro-oestrus, the day following mating being designated Day 1 post coitum (p.c.). Fetuses were recovered on Days 12, 13, 14, 15 and 16 p.c. and the fetal gonads were removed from the fetuses under a Leitz Stereo II dissection microscope using aseptic conditions. When animals were mated on the evening of pro-oestrus and allowed to give birth, the day of birth was referred to as Day 1 post partum (p.p.).

3.1.2 Rats

Rats (Rattus norvegicus) of a highly inbred Liverpool Hooded strain were obtained from the Medical Research Council Laboratory Animal Centre, Carshalton, Surrey, U.K. The colony was kept by mating brothers and sisters. The rats were maintained in conditions of controlled daylength (14 hours daylight and 10 hours darkness), and fed MacGregor Rat Cake 41B (Edinburgh, Scotland), and water ad libitum. Adult rats were mated and a vaginal smear was taken from

each female rat on the following morning to examine for spermatozoa as evidence of mating. The day following mating was designated as Day 1 p.c.. In cases where animals were allowed to give birth, the day of birth was referred to as Day 1 p.p.. Fetuses on Days 12 to 21 p.c. were recovered by killing the pregnant animals.

3.2 Determination of the sex of the fetuses

The sex of the fetus was determined from the gonadal sex, the presence of sex chromatin bodies in liver cell nuclei from hamsters, or from the chromosomal karyotype taken from air-dried amniotic membranes.

3.2.1 Gonadal sex

When the gonads were excised after sexual differentiation, their sex was determined by preparing histological sections from one gonad from each fetus. When the gonads were excised before gonadal sexual differentiation, one gonad from each fetus was cultured as a control, and its sex was determined at the end of the culture by examining histological sections (for histology methods, see section 3.6).

3.2.2 Sex chromatin

The determination of the presence or absence of sex chromatin in cells was found to be applicable only to hamsters. Sex chromatin was easily distinguished in hamster liver cells. The hamster fetus was dissected and the liver was removed, cut and washed with normal

saline to remove blood. The cut end of the liver lobe was scraped with a glass spatula and the smear was transferred onto a clean glass slide which was then immersed immediately in carbowax fixative (Industrial spirit, 100 ml; polyethylene glycol 1500, 3 gm; glacial acetic acid 0.3 ml) for at least 15 minutes. The slide was then passed through graded alcohol into water and was stained by 0.5% aqueous creysl violet (Raymond A. Lamb, London, U.K.). The smear was then dehydrated and mounted with DPX. The slide was examined under an oil immersion lens and at least 50 cells were scored from each fetus.

3.2.3 Chromosomal preparations

The air-dried chromosomal preparations from rat and hamster embryos were made by the technique by Evans, Burtershaw and Ford (1972). The amniotic membrane and the tail-tip of the embryo were used. The medium used was Eagle's minimum essential medium with Hank's balanced salts.

After the preparation of suitable slides, the chromosomes of the cells in metaphase were examined under oil immersion and the sex chromosome was identified.

In the rat, the Y chromosome can be identified as the samllest acrocentric chromosome while in the hamster, the X chromosome is the largest submetacentric chromosome which can be identified easily.

3.3 ORGAN CULTURE OF HAMSTER GONADS

Hamster fetuses on Day 12 to Day 16 p.c. were removed with aseptic techniques. The urogenital complex consisting of the gonads together with the attached cranial part of the mesonephros and the rete ovarii, was dissected from the fetus under sterile conditions. The rete ovarii enters the gonad cranially and joins the sex cords; ovaries without rete ovarii were therefore obtained by trimming away the cranial part of the ovary together with the rete tubules.

3.3.1 Culture methods

The technique of organ culture was essentially that described by Baker and Neal (1969, 1973). The gonads were placed on blocks of 2% agar in plastic Petri dishes. The culture medium consisted of Eagle's minimum essential medium with Earle's salts, buffered with bicarbonate, and was supplemented with newborn calf serum (20%v/v)., L-glutamine (2mM/L) and antibiotics (Amphotericin B, 1.5 µg/ml, Kanamycin, 30 µg/ml) (all the media used were obtained from Flow Laboratories, Irvine, U.K.). The Petri dishes were placed in modified 'Kilner' jars which were gassed at 0·703 kg/cm^2 with 5% CO_2 in air and were incubated at 37°C. The culture medium was replaced every 3rd or 4th day.

At the end of the period of organ culture, the gonads (still attached to agar blocks) were fixed in Bouin's aqueous fixative prior to histological processing.

3.3.2 Relationship between the rete ovarii and ovarian development

Female hamsters were sacrificed and fetuses were recovered on Days 12, 13, 14, 15 and 16 p.c.; the sex of each fetus was determined from the karyotype or from the presence of sex chromatin bodies in liver cell nuclei. Ovaries with or without rete were maintained in culture as shown in Table 2. Ovaries at the end of the culture were fixed in Bouin's fluid.

3.3.3 Relationship between the rete ovarii and testicular development

Fetuses were recovered on Days 12, 14, 15 and 16 p.c.; the sex of each fetus was determined. Fetal testes were cultured with ovaries (with or without rete) to investigate the effect of the external rete ovarii on the initiation of meiosis in the testis. As a control, two testes from fetuses of similar age were cultured together. The experimental design is shown in Table 3. At the end of the culture, the gonads were fixed in Bouin's fixative and processed for histological examination.

3.3.4 Culture of testis separated by a millipore filter from an ovary with rete ovarii.

Gonads were excised from Days 14, 15 and 16 p.c. fetuses. To set up the culture, the gonad was placed on a piece of millipore filter on top of an expanded steel grid, ovaries and testes were placed on opposite sides of another filter as shown in Fig. 2. The millipore filter used had a pore size of 0.15 µm. At the end of the culture, the gonads were fixed together with the filters. Table 4 summarises this experiment.

Table 2: Culture of fetal hamster ovaries with or without rete ovarii

	Gestational age at culture (days p.c.)				
No. of cultures	12	13	14	15	16
Ovary with rete	4	4	5	5	3
Ovary without rete	4	4	5	5	3
Duration of culture (days)	6-16	5-14	4-12	3-10	3-10

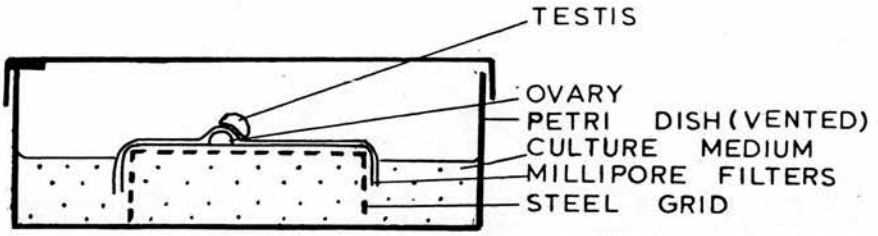
Table 3: Culture of fetal hamster testes with ovaries (with or without rete ovarii)

	Gestational age at culture (days p.c.)			
No. of cultures	12	14	15	16
Ovary with rete + testis	6	8	3	6
Ovary without rete + testis	4	-	-	3
Testis + testis	4	9	9	6
Duration of culture (days)	6-16	4-12	3-10	3-10

Table 4: Culture of fetal hamster testis separated by a millipore filter from an ovary with rete ovarii

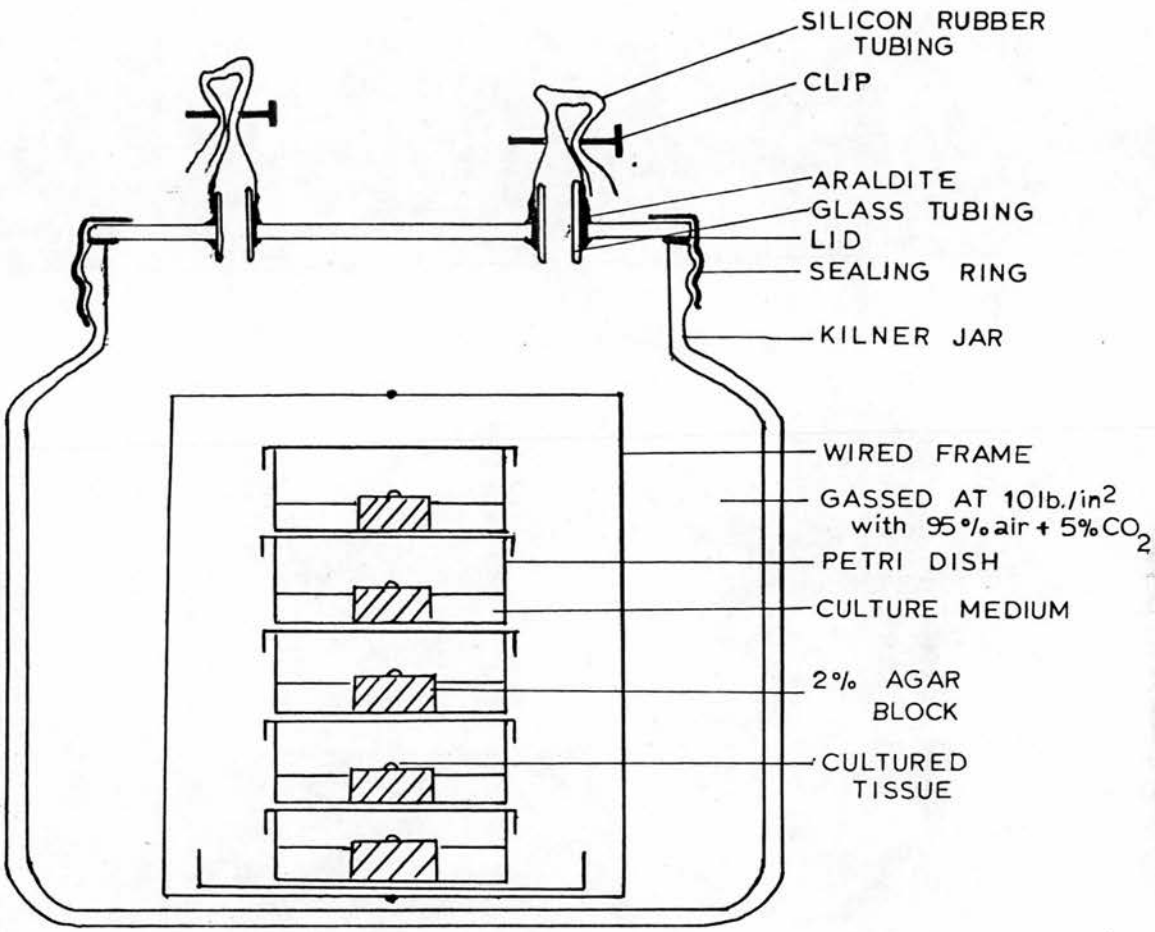
Sex of gonads	Gestational age (days p.c.)		
	14	15	16
M / F	2	3	3
F / M	2	2	1
F / F	2	2	2
M / M	2	1	2
Duration of culture (days)	3-10	4-8	3-12

M = testis; F = ovary; ___ = millipore filter



2 X ACTUAL SIZE

FIGURE 2.A CROSS SECTIONAL VIEW TO SHOW THE ORGAN CULTURE OF A TESTIS SEPARATED BY A MILLIPORE FILTER FROM AN OVARY.



ACTUAL SIZE

FIGURE 2.B CROSS SECTIONAL VIEW OF THE MODIFIED KILNER JAR USED IN ORGAN CULTURE.

3.4 TRANSPLANTATION OF RAT GONADS UNDER THE KIDNEY CAPSULE OF ADULT CASTRATED RATS

Liverpool hooded rats were used in the transplantation experiments. The donors and the hosts all came from the same parents.

3.4.1 Castration

Adult rats were anaesthetised in an ether jar, and were kept anaesthetised during the operation with the aid of an ether cone. For male castration the hind legs were tied down and the hair overlying the scrotal area was clipped. The skin was cleaned with 2% Cetavlon. A medial incision was made through the skin of the scrotum. A second incision was then made through the transparent tunica vaginalis exposing the testis and the epididymis. A ligature was passed around the spermatic cord, and the testis was removed by a cut close to the ligature. The second testis was removed in the same manner. The skin incision was closed with Michel clips.

To ovariectomise the female rats, the abdominal hair was clipped over the ventral abdomen and the area was cleaned with 2% Cetavlon. The animal was laid on its back and a mid-line incision about 2 cm long was made. The viscera were gently moved to one side, exposing the uterus and ovary. The fat was withdrawn and the ovary was separated and ligated with a silk ligature. The ovary was then cut away. The ovary on the opposite side was then removed in a similar manner. The body wall was closed using interrupted mattress sutures, and the skin was closed by Michel clips.

3.4.2 Transplantation of fetal gonads under the kidney capsule of castrated hosts.

Fetal gonads aged from Day 12 to Day 21 p.c. were recovered from pregnant rats under aseptic conditions. Male and female rats castrated for at least 14 days were used as recipients of the fetal gonads. The animals were anaesthetised with ether and hair was clipped from the back. The area was cleaned with 2% Cetavlon and an incision of about 1.5 cm long was made in the skin, about 1 cm lateral to the vertebral column and about 1 cm posterior to the last rib. A second incision through the muscle layers exposed the kidney. The kidney was gently lifted up with a blunt seeker which also served to keep the kidney outside the body wall in position for the transplantation. On the flat side of the kidney surface, the kidney capsule was punctured near the edge with a Gauge-21 needle, previously moistened with normal saline. A tunnel of about 8 mm was made using the needle just underneath the kidney capsule, and the needle was withdrawn. One gonad of each sex was transplanted to each host kidney in such a position that the explants were located side by side in contact. The gonads were introduced to the end of the tunnel using a glass capillary pipette drawn to the suitable diameter. When the supporting seeker was removed, the kidney slipped back into the body cavity and the skin was closed by Michel clips. The transplantation procedure was also repeated on the other side of the kidney. Two gonads of the same sex were transplanted to each host kidney as a control. Gonads were also transplanted under the kidney capsule of intact hosts for comparison. At the end of the experiment, the hosts were killed and the donor tissues together with the host kidney were fixed in Bouin's fixative for about 18 hours. Table 5 is the summary of the transplantation experiments.

Table 5: Transplantation of fetal rat gonads into adult hosts

Sex of host	Age (days)	Sex of donor gonads ♀♂	♂♂	♀♀	equivalent age recovered (days)
4⚲; 2⚲	12 pc	5	1	1	5 pp
6⚲; 4⚲	14 pc	5	4	6	7 pp
4⚲; 3⚲	16 pc	5	5	3	9 pp
4⚲; 2⚲	17 pc	5	2	1	10 pp
3⚲; 3⚲	20 pc	4	2	3	14 pp
6⚲	3 pp	4	3	2	17 pp
3⚲	16♂ + 14♀ pc	4	-	-	-
3⚲	14♂ + 16♀ pc	4	-	-	-
5♂; 1♀	14 pc	3	2	5	7 pp
2♂; 3♀	18 pc	5	4	4	11 pp

⚲ = castrated male host

⚲ = castrated female host

3.5 CHIMAERIC GONADS

The method for the separation of germinal and somatic cells was modified from the method by Steinberger and Steinberger (1965). Gonads were removed from fetuses using sterile techniques. The fetuses were sexed by the sex-chromatin method. Gonads of the same sex were pooled together, and each was cut into segments not larger than 1 mm in size in 5 ml phosphate buffered saline (Dulbecco's formula, without magnesium and calcium) (Flow Laboratories, Irvine, U.K.) warmed up to 32°C. 0·2 mg of DNAase (Sigma, U.K.) and 5 ml of 0·5% freshly made up trypsin solution (in phosphate buffered saline) was added to the suspension to give a final concentration of 0·25% trypsin. The suspension was repeatedly agitated to enhance separation in the first 10 minutes. The tissue was left to be trypsinised for a further 10 minutes. Cell clusters at the end of trypsination were allowed to settle to the bottom of the flask and the supernatant containing single cells was spun at 900 rpm for 10 minutes. The cells were washed once with phosphate buffered saline and then with culture medium (Section 3.3.1) containing 20% newborn calf serum, and were then suspended in the culture medium.

Counts of viable cells were performed using 1% trypan blue dye in a haemocytometer. The ability to exclude the dye was used as the criterion of viability. The cell suspension was then divided into aliquots containing about 10^6 cells and layered onto 18mm^2 No. 3 glass coverslips previously cleaned in absolute alcohol, dried and heated to 200°C. The coverslips were placed into tissue culture chambers containing twenty-one 19mm^2 compartments. Each compartment received 2 ml culture medium. The suspension was cultured at 37°C for at

least 18 hours in a stationary position in air. The medium with the cells not adhering to the culture compartment was pipetted out and spun down at 900 rpm for 10 minutes. The supernatant was removed and the cells were resuspended in fresh culture medium; these were the germinal cells of the fetal gonads.

The glass coverslips were lifted from the culture comparments, washed twice with phosphate buffered saline to remove culture medium and calf serum, and were then incubated for 10 minutes in 0·25% trypsin solution. The trypsin solution was then centrifuged and the cells washed twice in culture medium. This gave the non-germinal (or somatic) cell fraction of the gonads.

The separated germinal and non-germinal elements were counted and tested for viability. The male germinal cells were then mixed with the female somatic cells, and centrifuged at 900 rpm for 15 minutes in a conical centrifuge tube. The pellet, after centrifugation, was lifted up by a glass pipette, and transferred onto an agar block (2% agar made up in distilled water and equilibrated overnight in culture medium). Similarly, chimaeric gonads were made with female germinal cells and male somatic cells. The chimaeric gonads were then cultured according to the method described in section 3.3.1. Chimaeric gonads at the end of the culture were fixed and processed for histological examination.

The number of chimaeric gonads prepared is summarised in Table 6.

Table 6: Culture of fetal hamster chimaeric gonads

Fetus			Chimaeric gonad		
Age (pc)	Sex	No.	XXSoma/XYGerm	XYSoma/XXGerm	Days in culture
12	♂	24	1	1	3
	♀	20			
12	♂	23	1	1	8
	♀	28			
13	♂	19	1	1	3
	♀	26			
13	♂	24	1	1	6
	♀	18			
14	♂	21	1	1	3
	♀	18			

3.6 HISTOLOGY

All the tissues were fixed in Bouin's fixative for 3 to 18 hours according to the size of the tissue, and were then changed into 70% alcohol. They were then dehydrated and embedded in paraffin wax (M.P. 56°C). Serial sections, cut at a microtome setting of 5 um, were deparaffinized and stained with Harris' Haematoxylin and eosin stain or with Heindenhain's azan stain.

Sections were examined, and the stages of meiotic division in the oocytes were identified. The seminiferous tubular diameter was measured (the longest and the shortest axis of the tubular cross sections), and the interstitial Leydig cell size was also measured (the longest and the shortest axis of the cell and the nucleus). All the measurements were done with the Leitz micrometer eyepiece.

Chapter IV IN VITRO CULTURE OF HAMSTER GONADS

4.1 Introduction

It has been well established that fetal gonads, when excised and maintained in suitable conditions, can differentiate normally (Wolff and Haffen, 1965). However, if the ovaries are removed from the fetuses before the onset of meiosis, the germ cells fail to undergo normal meiosis in culture and subsequently degenerate (mouse: Wolff, 1952; sheep: Mauléon, 1973; hamster: Challoner, 1975a).

In the majority of female mammals, meiosis begins before birth (rat: Beaumont and Mandl, 1962; mouse: Borum, 1961; rhesus monkey: Baker, 1966; man: Baker, 1963; sheep: Mauléon, 1963) while in others meiosis begins immediately after birth (rabbit: Peters, Levy and Crone, 1965; hamster: Challoner, 1974; ferret: Deansley, 1970). Since meiosis does not begin until after birth in the hamster, it is easy to obtain fetal ovaries that are at a relatively early stage of differentiation.

In the hamster, sexual differentiation has been studied by various authors (Ortiz, 1945; Price and Ortiz, 1965; Gondos, Paup, Ross and Gorski, 1974). The main events are summarised in Fig. 4.1. It has been shown that meiosis fails to occur in hamsters if the ovaries are removed before the 15th day of gestation even if gonadotrophins or maternal serum are added to the cultures, suggesting that an inductor is required to initiate meiosis (Challoner, 1975a). However, the meiosis inducing factor has not been investigated further. Byskov (1974) grafted fetal mouse ovaries into nude mice and showed that the external rete ovarii was necessary to trigger meiosis in the germ cells. In the present study, the effect of the external rete ovarii on the female germ cells in the hamster was studied in organ culture.

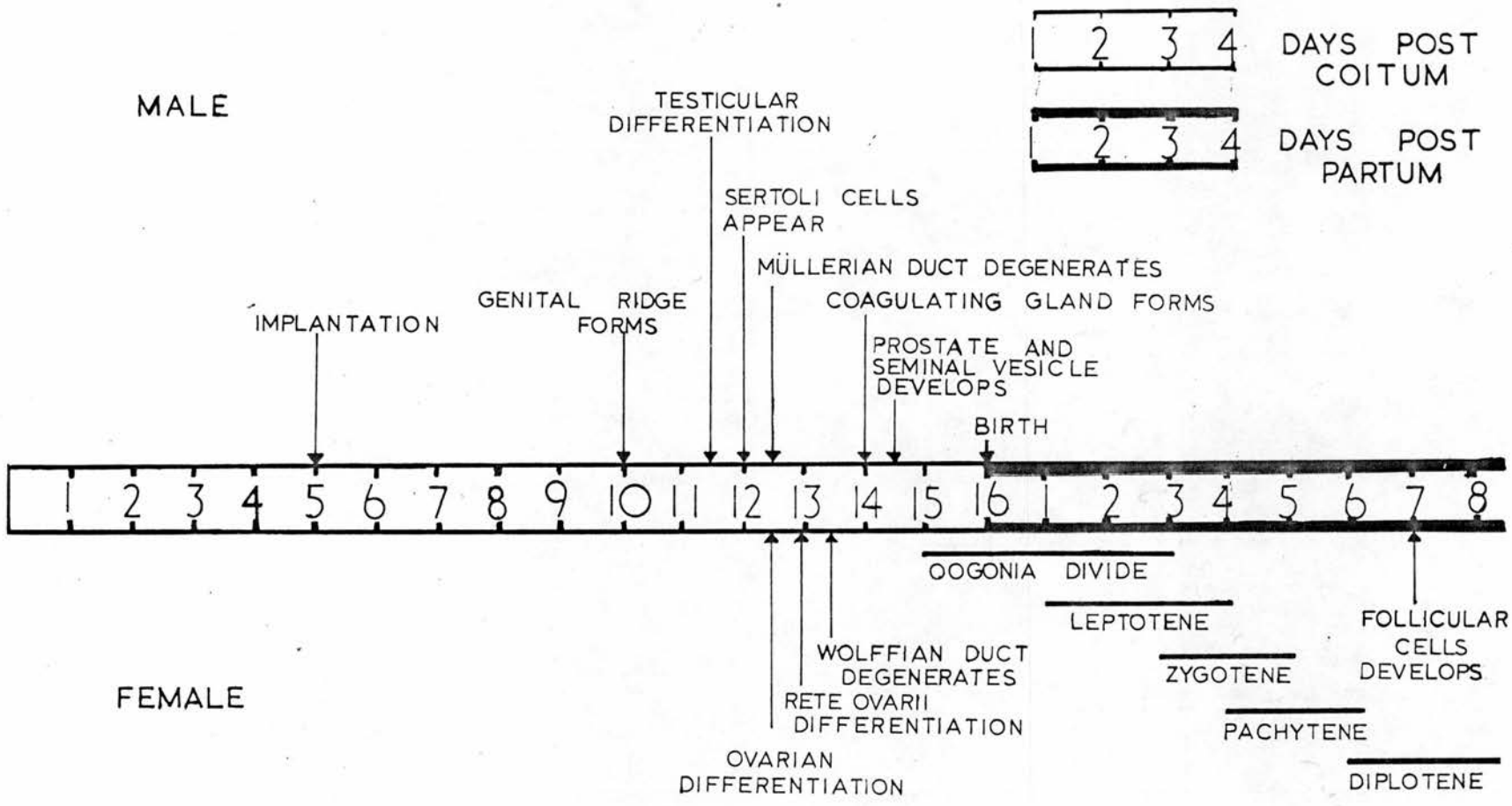


FIGURE 4.1 SEXUAL DIFFERENTIATION IN THE HAMSTER

In the male, germ cells will not start meiosis until puberty. It has been well established that pituitary gonadotrophins and particularly FSH are required in addition to testosterone to initiate spermatogenesis at puberty. This appears to be related to a specific effect of FSH on the Sertoli cells of the testis, causing them amongst other things to synthesise androgen-binding-protein (Hansson, Trygstad, French, McLean, Smith, Tindall, Weddington, Petrusz, Nayfey and Ritzen, 1974). However, very little is known about the control of male germ cell differentiation. All efforts to get mammalian spermatogenesis to proceed in organ culture beyond the pachytene spermatocyte stage have so far failed (Steinberger, Steinberger and Ficher, 1970).

Whether the factor that initiates meiosis is the same in both the male and the female remained to be tested. This problem was investigated by culturing fetal testis in contact with fetal ovary together with external rete ovarii. Cultures of fetal hamster testis separated by a millipore filter from an ovary with rete ovarii were also studied.

4.2 Material and Methods

The animals used and the techniques of culture were described in 3.1.1 and 3.3.1.

The sex of the hamster fetuses before Day 13 p.c. was determined by the presence or absence of sex chromatin in the liver cells (see section 3.2.2); 50 cells from each fetus were scored under x1000 magnification.

Gonads from Day 12 to Day 16 p.c. hamster fetuses were examined histologically as a normal control to compare with the cultured

controls. The stages of meiosis in the ovaries were identified according to Challoner (1974), and the development of the ovary and testis was based on the work of Nakano (1960).

The establishment of the cultures was described in sections 3.3.2, 3.3.3 and 3.3.4. The cultured gonads were processed for histological examination, and two representative sections from each culture were scored for the total number of germ cells, the number of germ cells undergoing meiosis, and the percentage of germ cells undergoing meiosis.

4.3 Results

Gonadal sex differentiation in the golden hamster begins on Day 12 p.c. (see Fig. 4.1) and is marked by the formation of the tunica albuginea in the testis. This is not clearly recognisable until Day 13 p.c., therefore fetuses on Day 12 p.c. have to be sexed by looking for sex chromatin in the female liver cells. Hamster liver cell nuclei are very granular and sex chromatin appears as a hemispherical, darkly staining structure on the nuclear membrane (Plate 1.1). Fetuses with more than 60% of the liver cells containing sex chromatin were identified as females.

4.3.1 Gonadal development in vivo

The development of the hamster testis and ovary, together with a description of the various stages of meiotic prophase, were ~~described~~ provided by Nakano (1960) and Challoner (1974), and therefore only a brief account needs to be given.

The ovary and testis are morphologically indistinguishable before Day 12 p.c.. On Day 12 p.c. a primitive tunica albuginea (Plate 1.2) can be seen in the male separating the coelomic epithelium from the inner medullary region.

On Day 13 p.c., seminiferous tubules become recognisable in the testis (Plate 1.3). In the ovary, mitotic divisions of the germ cells can be observed. The rete ovarii can be found as accumulations of epithelial cells in the hilar region extending towards the mesovarium (Plate 1.4).

On Day 14 p.c., Leydig cells can be distinguished between the seminiferous tubules of the testis. In the ovary, the oogonia

continue to proliferate and they are now arranged in cords separated by a single squamous epithelium. The rete ovarii joins the germ cell cords.

On Day 15 and 16 p.c., both the ovary and the testis continue to proliferate. Mitotic divisions of oogonia continued until on Day 1 p.p. when meiotic prophase starts. By Day 3 p.p., most of the oogonia have reached leptotene or zygotene. Two days later, about 80% of the oocytes are at pachytene, and oocytes at diplotene can be seen after Day 5 p.p. Male germ cells do not undergo meiosis until shortly before puberty.

4.3.2 Culture of ovaries with and without rete ovarii

Results of this experiment were summarised in Table 4.1.

A. Culture of ovaries removed from fetuses on Day 12 and Day 13 p.c.

In the culture of the cranial part (with rete ovarii) and the caudal part (without rete) of the Day 12 p.c. ovary, oogonia undergoing meiosis were observed in ovaries fixed 4 to 5 days after the onset of culture. Two days later, in the ovary with rete, the nuclei of most of the oogonia attained a very grainy appearance, which was considered to be the preleptotene stage of meiotic prophase (Plate 1.5). The cultures fixed after different periods of culture showed that the oogonia progressed through different stages of meiotic prophase. After 14 days in culture, they reached the diplotene stage of the meiotic prophase (Plate 1.6), but the organisation of the follicular cells was poor as compared with a normal ovary at an equivalent stage of development, namely Day 9 p.p.

Table 4.1: Role of rete ovarii in oogenesis in organ cultures of hamster ovaries

	Gestational age at culture (days p.c.)				
	12	13	14	15	16
Duration of culture (days)	6-16	5-14	4-12	3-10	3-10
Ovary with rete					
No. of cultures	4	4	5	5	3
No. of ovaries with meiosis*	4	4	5	5	3
Ovaries without rete					
No. of cultures	4	4	5	5	3
No. of ovaries with meiosis*	0	0	5	5	3

* At least 90% of surviving germ cells undergoing meiosis.

Plates 1.1 to 1.6

1.1 Liver cells smear from a Day 12 p.c. female hamster to show the sex chromatin on the nuclear membrane (arrowed). Cresyl violet, x 1400.

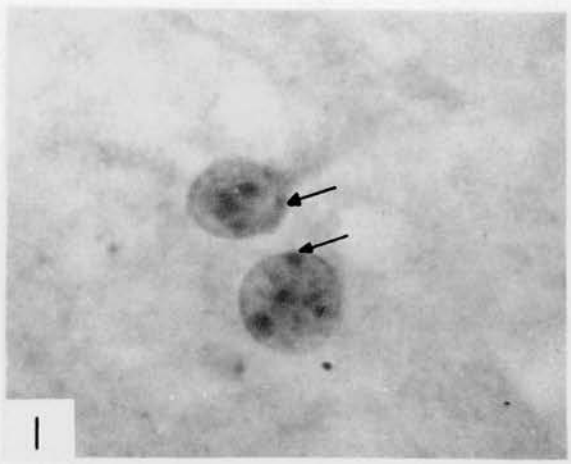
1.2 Day 12 p.c. hamster testis. Germ cells are in the medullary region and the tunica albuginea (t) is found underneath the coelomic epithelium (c). H.&E., x86.5.

1.3 Day 13 p.c. hamster testis. Seminiferous tubules (arrowed) containing germ cells (G) can be distinguished. H. & E., x210.

1.4 Day 13 p.c. hamster ovary (whole mount) to show the rete ovarii (ro) which is a group of coiled tubules in the hilar region. The mesonephros (m) is along the length of the gonadal ridge (gr). x 86.5.

1.5 Day 12 p.c. hamster ovary(with rete ovarii) after two days in culture. Germ cells are enclosed in cords with oogonia at preleptotene stage of meiotic prophase. H. & E., x840.

1.6 Day 12 p.c. hamster ovary (with rete ovarii) after 14 days in culture (equivalent to 10 days p.p.). Germ cells reach the diplotene stage of meiosis. Follicular cells organisation is poor. H. & E., x500.



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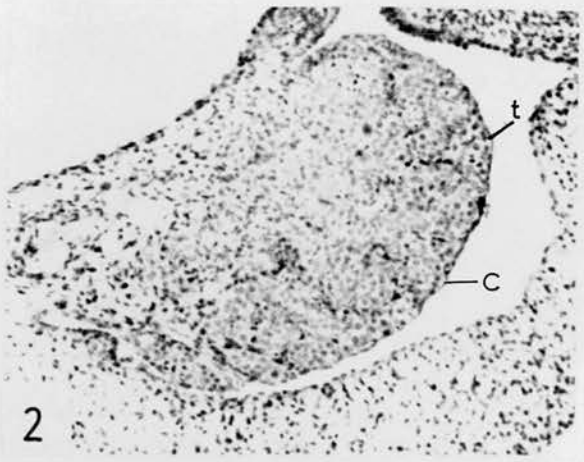

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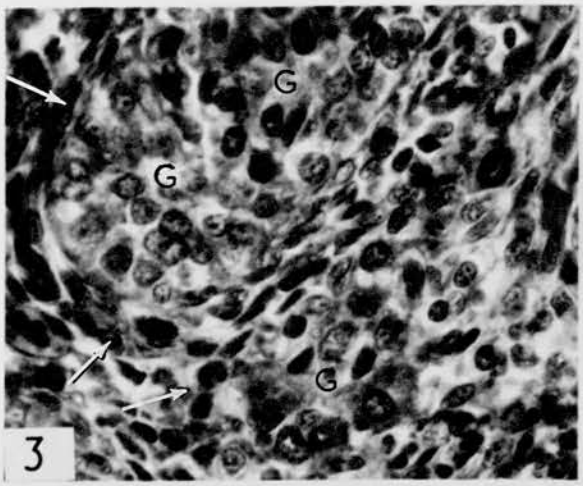

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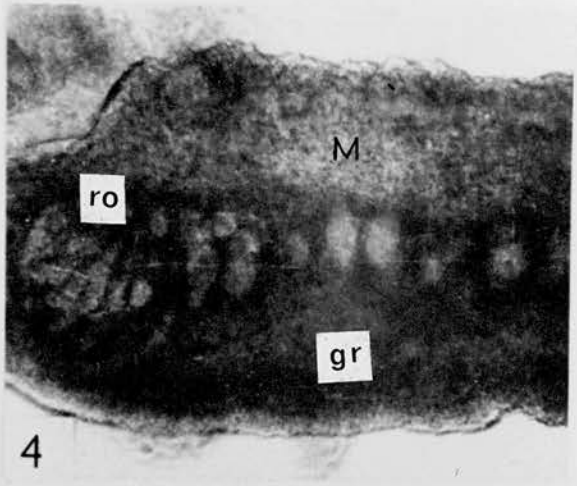

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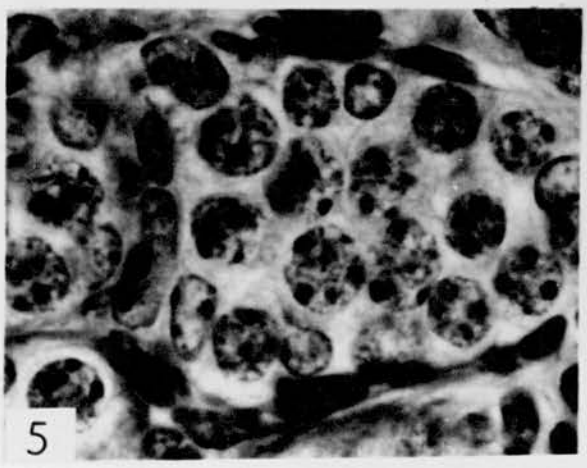

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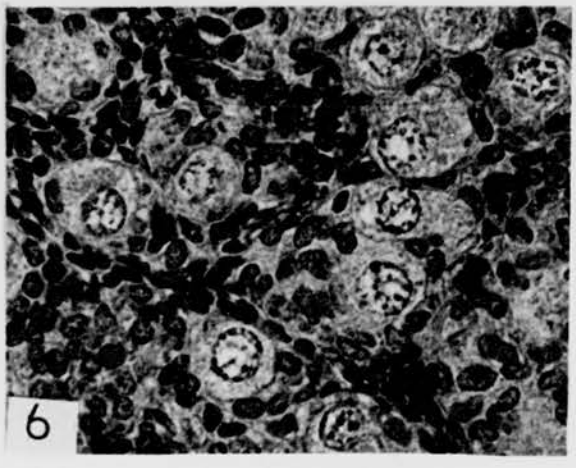


6

In ovarian cultures without the rete, the majority of the oogonia did not enter meiosis after 6 days in culture, although a few cells could be observed at leptotene. After about 8 days in culture (equivalent to Day 4 p.p.) a dramatic increase in the number of atretic oogonia and oocytes was found, and by the 12th day, the ovarian tissue was almost devoid of germ cells (Plate 1.7).

Similar results were observed in ovaries (with or without rete) removed from fetuses on Day 13 p.c..

B. Culture of ovaries removed from fetuses on Day 14, 15 and 16 p.c.

No difference was observed between cultures with and without rete. The histological appearance of ovaries maintained in organ culture was essentially similar to that of the controls in vivo: large numbers (>90%) of the germ cells entered the leptotene stage of meiosis; after 8 days in culture, the ovaries contained germ cells that had progressed through to the pachytene or diplotene stage (Plate 1.8).

The surviving germ cells were normal in appearance, but their numbers were much reduced compared with the in vivo controls.

4.3.3. Culture of fetal hamster testis with ovaries

Fetal testes were removed from fetuses on Days 12, 14, 15 and 16 p.c., and were maintained in culture as a control. The development of the testes in culture was similar to their normal development in vivo. The seminiferous tubules were well maintained and no meiosis was observed in the germ cells (Plate 1.9).

Table 4.2: Induction of meiosis in the fetal hamster testis cultured with or without a fetal hamster ovary in organ culture

	Gestational age at culture (days p.c.)			
	12	14	15	16
Duration of culture (days)	6-16	4-12	3-10	3-10
Testis + ovary with rete				
No. of cultures	6	8	3	6
No. of testes with meiosis+	6	8	3	6
No. of ovaries with meiosis*	6	8	3	6
Testis + ovary without rete				
No. of cultures	4	-	-	3
No. of testes with meiosis	0	-	-	3
No. of ovaries with meiosis*	0	-	-	3
Testis + testis				
No. of cultures	4	9	9	6
No. of testes with meiosis	0	0	0	0

* At least 90% of surviving germ cells undergoing meiosis.

\+ At least 30% of surviving germ cells undergoing meiosis.

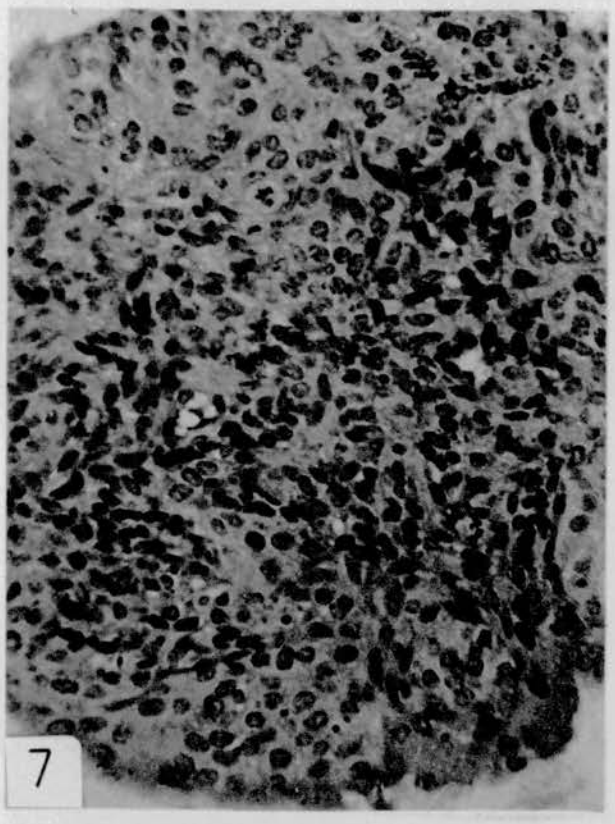
Plates 1.7 to 1.10

1.7 Ovary without rete ovarii recovered on Day 12 p.c. and cultured for 12 days. Only somatic cells are present in the ovarian tissue. H. & E., x 210.

1.8. Ovary without rete ovarii recovered on Day 14 p.c. and cultured for 8 days. Germ cells progress through to pachytene (P) or diplotene stage (D). H. & E., x 350.

1.9 Day 14 p.c. hamster testis cultured for 12 days. Seminiferous tubules and germ cells are well maintained. H. & E., x 350.

1.10 Day 14 p.c. hamster testis cultured for 4 days with an ovary of the same age. Testicular tubules are disorganised with considerable increase in somatic cells. H. & E., x 210.



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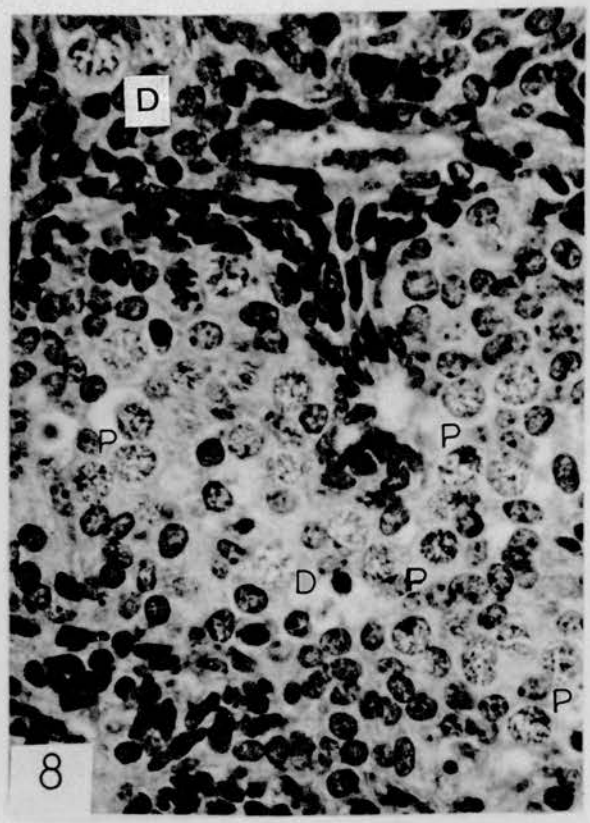

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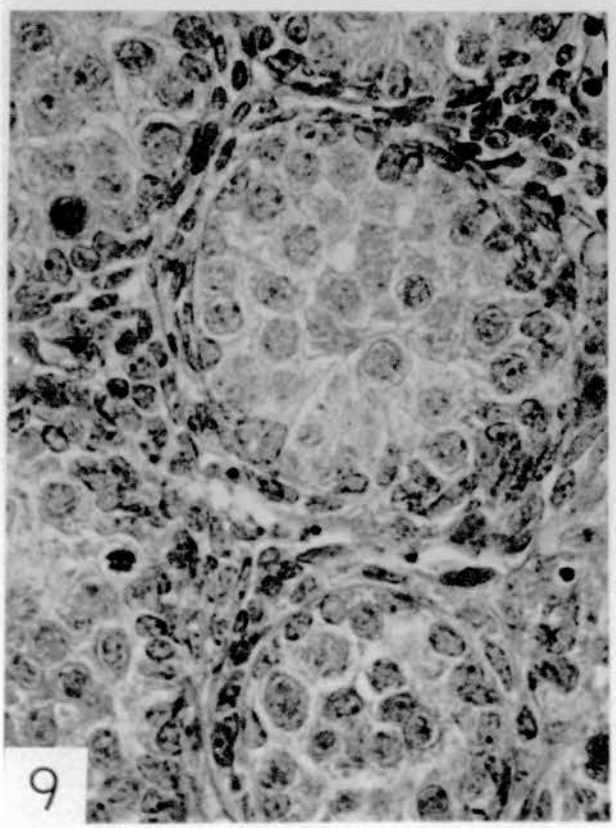

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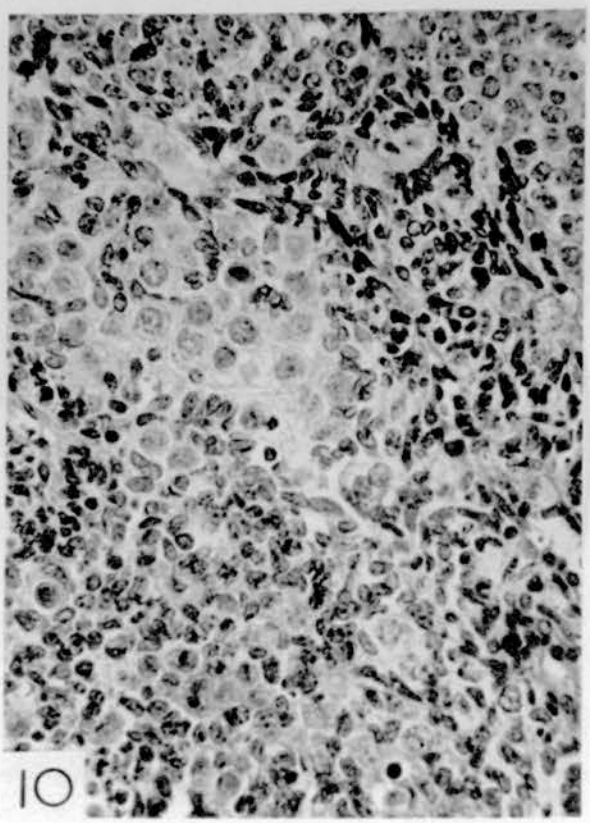

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A. Culture of fetal hamster testis together with a fetal ovary and its rete ovarii.

The testis and the ovary when cultured in close apposition became fused together as they proliferated. With gonads removed from fetuses on Day 12 p.p., after 8 days in culture the oogonia in the ovarian part entered meiosis, as described in section 4.3.2A. However, in the testicular part, the tubular structure was disorganised after 4 days in culture (Plate 1.10), although it could still be recognised by groups of gonocytes (Plate 1.10). Somatic cells proliferated and surrounded the germ cells either in groups or individually (Plate 1.11). These isolated cells entered meiosis precociously and some reached the diplotene stage of meiotic prophase after 14 days in culture.

Similar results were obtained in cultures set up from Day 14, 15 and 16 p.c. gonads, and these are summarised in Table 4.2.

B. Culture of fetal hamster testis with fetal ovary devoid of rete ovarii.

In the Day 12 p.c. fetal testis when cultured with an ovary (without rete) of the same age, the oogonia did not enter meiosis and became atretic, as described in section 4.3.2A. The testicular part of the culture was comparable to the testis-testis control cultures, with well maintained seminiferous tubules and no meiosis in the germ cells.

However, in the cultures from Day 16 fetuses, the oogonia went through meiotic prophase and reached the diplotene stage of division. After 4 days in culture, the seminiferous tubules in the

testicular part started to disorganise, but a lower percentage (less than 20%) of the male germ cells entered meiosis (Plate 1.12).

4.3.4 Culture of ovaries and testes separated by a millipore filter.

The millipore filter had a pore size of 0.15 μm; cellular contact between the testicular and ovarian part of the culture was not possible.

When testis and ovaries were placed on either side of the millipore filters, results were identical to those obtained when the gonads were grown in direct contact with one another. The findings are summarised in Table 4.3.

The ovary-ovary and the testis-testis cultures formed the controls, where growth was similar to that of normal gonads in vivo.

In the ovary-testis cultures, ovarian development was found to be normal with germ cells undergoing meiotic division (Plate 1.13). In the testis, which was placed directly above or underneath the ovary on the other side of the Millipore filter, the testicular tubules ~~was~~ were not well maintained and germ cells in different stages of meiotic prophase were present in the disrupted tubules (Plate 1.14). In all cases, about 20% of the male germ cells entered meiosis. The results indicate that the ovarian influence on modifying testicular development had diffused across the Millipore filter.

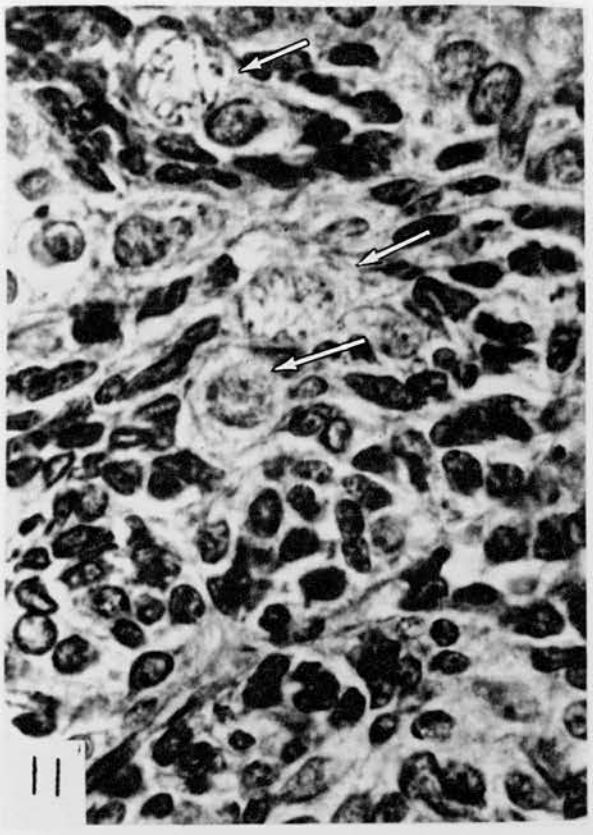
Plates 1.11 to 1.14

1.11 Day 14 p.c. hamster testis cultured for 12 days with an ovary of the same age. Note the large numbers of somatic cells and the male germ cells (arrowed) undergoing meiosis. H. & E., x350.

1.12 Day 16 p.c. hamster testis cultured for 6 days with an ovary of the same age. The testicular tubules disorganise and the male germ cells (arrowed) become isolated and their nuclei attain a 'grainy' appearance. H. & E., x350.

1.13 Day 14 p.c. hamster ovary cultured for 8 days with a testis separated by a millipore filter. Germ cells at pachytene (p) and diplotene (d) stages can be found. H. & E., x210.

1.14. Day 14 p.c. hamster testis cultured with an ovary separated by a millipore filter (F) for 8 days. The seminiferous tubules are disorganised. H. & E., x 140.



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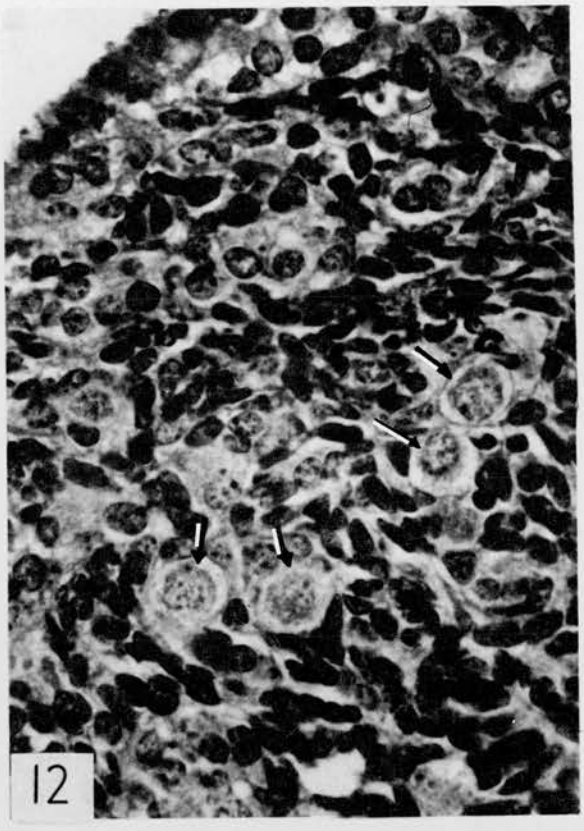

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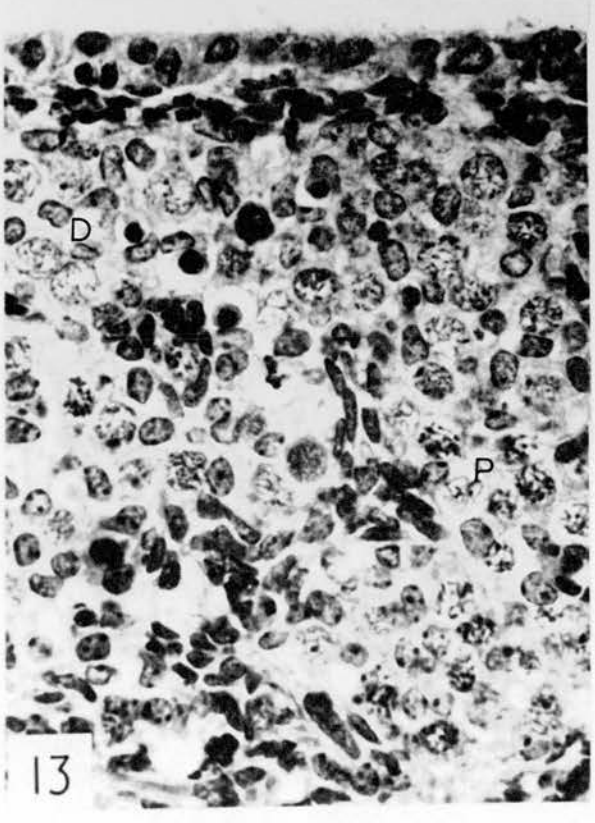

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14

Table 4.3: Culture of fetal hamster testis separated by a millipore filter from an ovary with rete ovarii.

Sex of gonads	Gestational age (days p.c.)		
	14	15	16
CONTROL			
OR / OR	2** / 2**	3** / 3**	2** / 2**
T / T	2 / 2	1 / 1	2 / 2
EXPERIMENT			
T / OR	2* / 2**	3* / 3**	3* / 3**
OR / T	2** / 2*	2** / 2*	1** / 1*

OR = ovary with rete ovarii

T = testis

—— = millipore filter

* = about 20% germ cells entering meiosis

** = more than 90% germ cells entering meiosis

n = nos. of cultures

4.4 Discussion

There have been three important discoveries in this study: (i) the rete ovarii is essential for the initiation of meiosis in female hamster germ cells; (ii) male fetal hamster germ cells when cultured together with an ovary with its rete ovarii can enter meiosis precociously; (iii) the "meiosis-inducing factor" which emanates from the rete ovarii, is a diffusible substance (see below).

4.4.1 Role of the rete ovarii in the initiation of meiosis in female germ cells.

The control mechanism for the onset of meiosis in mammals is poorly understood. It has been shown that germ cells in organ culture will survive and progress beyond the leptotene stage in ovaries which are excised after a certain critical stage of development. This critical stage seems to coincide with the time the somatic element, the rete ovarii, starts to differentiate (Byskov, 1975). The interaction between somatic and germinal cells during gonadal sex differentiation has been shown to be important in the determination of the fate of the gonad (Short, 1972; O and Short, 1977), and in the present study, the rete ovarii seems to be the first somatic element in the gonad that is directly involved in the regulation of germ cell development.

The importance of the rete ovarii in the control of meiosis in female germ cells was first demonstrated in transplantation experiments (Byskov, 1974) where Day 12 to Day 16 p.c. mouse ovaries were grafted subcutaneously into nude mice with or without rete ovarii;

it was shown that the rete ovarii was essential for Day 12 ovaries to enter meiosis and form follicles. The present study, as reported by O and Baker (1976), conclusively shows that the external rete ovarii is involved in the initiation of meiosis, because even when the ovaries and rete are maintained in vitro, the influence of the rete is still observed.

Similar observations have now been made in organ cultures of Day 12, 13 and 14 p.c. rat ovaries (Rivelis, Prepin, Vigier and Jost, 1976). The authors suggested that it was the mesonephros, rather than the rete ovarii, that triggered the process of meiosis. This recalls the argument about the embryonic origin of the rete ovarii, as discussed in section 1.5.1. Many authors believe that the entire rete blastema of the ovarian medulla is mesonephric in origin (Waldeyer, 1870; Wallart, 1928; Witschi, 1951) while others state that it is derived from mesenchymal tissue (Sauramo, 1954a) or from the surface epithelium. If the rete ovarii and the mesonephros are of the same embryonic origin, the argument about whether it is the rete or the mesonephros that initiates meiosis is unimportant.

4.4.2 Mode of action of the external rete ovarii

So far, it has been shown for the mouse, hamster and rat that the external rete ovarii is essential for the initiation of meiosis. Whether this depends on direct contact between the rete and the germ cells, or on a diffusible substance remains to be confirmed.

Secretory activity has been reported in the rete tubules of the dog (O'Shea, 1966), cat (Mossman, 1969) and heifer (Archbald, Schultz, Fahning, Furtz and Zemjamis, 1971). In the study of the

development of the rete ovarii in man (Sauramo, 1954a), mouse and hamster (Byskov and Lintern-Moore, 1973) and mink, cat and ferret (Byskov, 1975), open connections between the rete tubules and the epithelial cell cords containing the oogonia were observed. PAS-positive granules were also found in the rete cells around the time of commencement of meiosis in the germ cells (Byskov, 1975) and were thought to contain the meiotic inducing substance (MIS). The initiation of meiosis by a diffusible factor secreted from the rete can explain the situation in rodents where the onset of meiosis of germ cells takes place synchronously. However, in mammals with longer gestational periods, meiosis in the female germ cells occurs asynchronously over a long period of time (ca 7 months in cow and man) and it is hard to visualise how a diffusible factor can selectively affect germ cells which lie close together. This may suggest that the meiotic inducing factor must be a very weak inductor which acts only on the oogonia in close proximity to the rete cells. As intercellular bridges occur between adjacent oogonia as a result of incomplete cytokinesis during mitotic division (Gondos and Zamboni, 1969), the meiotic inducing substance could influence individual syncytial groups of germ cells without necessarily affecting neighbouring groups.

Another possible mode of action of the rete is by direct cellular contact. Transmission of morphogenic signals between embryonic cells can also occur by means of intercellular contact. It is known that cells from the spinal cord can cause the induction of mouse kidney cell development. When the nervous tissue and the kidney cells are separated by a membrane filter, induction only occurs if

cell-to-cell contact is established through the pores of the filter which are 0.2 um in diameter or larger; no evidence for a diffusible substance was found (Wartiovaara, Nordling, Lehton and Saxen, 1974). Cytoplasmic penetration into the filter was demonstrated by electron microscopy and by electron scanning techniques (Saxén, Lehton, Karkiner-Jääkeläiner, Nordling and Wartiovaara, 1976). Whether the rete cells and the germ cells interact by direct cytoplasmic contact was investigated by culturing rete ovarii and fetal testis separated by a millipore filter of 0.15 μm pore size. The result indicates the existence of a diffusible factor because the 0.15 μm pore size does not allow cytoplasmic contact, but meiosis is still induced in a testis separated from the rete by a filter.

4.4.3 Initiation of meiosis in male germ cells.

In the male, meiosis normally does not occur until puberty. Although endocrine factors are thought to be responsible, little is known about hormonal control of male germ cell differentiation. All efforts to get mammalian male germ cells to proceed beyond the pachytene stage in culture have so far failed (Steinberber, Steinberger and Ficher, 1970).

In XX/XY chimaeric mice, germ cells which have entered meiotic prophase were occasionally observed in fetal testes (Mystowska and Tarkowski, 1970; McLaren, Chandley and Kofman-Alfaro, 1972). MacLaren (1972) proposed that the germ cells undergoing meiosis might be XX as well as XY cells that were triggered to enter meiosis by surrounding XX somatic cells. The present study showed that male fetal germ cells were capable of entering meiosis precociously under the influence of the rete ovarii, thus supporting this suggestion.

Byskov and Saxen (1976) cultured fetal mouse testes and ovaries (with rete) from various stages of gestation, separated from one another by a Millipore filter. They found that when an undifferentiated heterosexual pair of gonads were cultured together, the male germ cells were triggered to enter meiosis; when older fetal testes, in which the testicular cords had already developed, were cultured with ovaries of the same age, the oocytes in the ovary were prevented from reaching diplotene. The authors concluded that both the male and the female secrete diffusible substances from the rete which affect germ cell differentiation. They proposed that the male rete cells secrete a "meiosis-preventing substance" (MPS) which can arrest the female cells at meiotic prophse, while the female gonad secretes a "meiosis-inducing substance" (MIS) which can stimulate undifferentiated male germ cells to enter meiosis.

The present study in the hamster thus confirms the presence of a MIS which is secreted by the rete ovarii. However, the MPS effect has not been demonstrated in cultures of older gonads. Species differences may be one possible explanation. It may not be necessary to postulate a meiosis-preventing substance to explain normal testicular differentiation. Perhaps the male germ cells do not enter meiosis during fetal development simply because the rete testis does not secrete the MIS; another controlling mechanism, mediated by hormones, may be involved in triggering meiosis at puberty.

The Sertoli cells seem to play an important role in the control of meiosis. A close association between the Sertoli cells and germ cells is established early in development, when the primitive cell cords can be recognised (Gondos, 1975). The regulation of testicular

growth by gonadotrophins seems to be mediated by the supporting cells: LH stimulates the interstitial cells of Leydig to synthesise androgen, mainly testosterone, and a high local concentration of androgen is essential for spermatogenesis; FSH stimulates Sertoli cells to synthesise an androgen binding protein (French and Ritzen, 1973) which contributes to the maintenance of a high androgen concentration in the seminiferous epithelium. In the period prior to the initiation of spermatogenesis, a sharp increase of DNA synthesis in the Sertoli cell nucleus has been reported (Okada, 1970; Ortavant, Courot, Hochereau-de Reviers, 1972). Occluding junctions form between the Sertoli cells before puberty to isolate the premeiotic germ cells from the meiotic and post-meiotic germ cells in the adluminal compartment of the seminiferous tubules (Gilula, Fawcett & Aoki, 1976).

In the present study, the disruption of tubular structure was seen in organ cultures of testis where precocious meiosis occurred. This could be due to the dislocation of the male germ cells from their Sertoli cells, thus disrupting the architecture of the seminiferous tubules. This is another example of possible germinal and somatic cell interaction.

4.4.4 Control of gonadal differentiation

In normal gonadal differentiation, female (XX) germ cells enter meiosis before male (XY) germ cells. This cannot be due to the difference in genotype of the germ cells, since it has been shown in the present study that both XX and XY germ cells can enter meiosis

simultaneously under appropriate experimental conditions. Therefore, this differential effect must be due to a somatic influence. This could be the meiotic inducing substance, which might be produced earlier by XX rete cells than XY ones. Whether the secretion of MIS depends on the presence of two X chromosomes, or whether the presence of a Y chromosome inhibits the secretion, remains to be determined. However, by considering various naturally occurring situations, as shown in Table 4.4, we may get some clues as to the answer.

Both X chromosomes may not be necessary to cause the rete cells to secrete MIS because XO mice and human fetuses with Turner's syndrome have ovaries with germ cells entering meiosis. Does the presence of a Y chromosome prevent the secretion of the MIS? In normal males, this appears to be the case. However, in the unusual situation of the XY female wood lemming, the rete cells must be secreting MIS even in the presence of a Y chromosome. This baffling situation can be explained because we believe that there is an X-linked Y-suppressor gene, so that it is as if there is no Y chromosome at all (Fredga et al., 1975). This idea is supported by the absence of the H-Y antigen (see section 1.3.3), in the XY female wood lemming (Wachtel, Koo, Ohno, Gropp, Dev, Fantavahi, Miller and Miller, 1976). The inhibition of MIS production by the Y-chromosome may also be blocked by the X-suppressor gene. In the case of XO, Sxr and XX, Sxr mice where the Y chromosome is absent, the rete testis should secrete the MIS. But Wachtel et al. (1975) showed that the cells from Sxr mice are H-Y antigen positive. This either implies that there has been an autosomal mutation, so that the Sxr can mimic the Y expression of H-Y antigen, or maybe there has been a Y-autosomal translocation (Cattanach, 1975).

Table 4.4: Relationship between the sex chromosomes of soma and germ cell fate

Genotype of somatic cells	Gonadal sex	Germ cells entering meiosis	Fate	References
XX	♀	+	oocyte	Normal development
XY	♂	+	spermatocyte	
	♀	+	oocyte	wood lemming: Fredga et al. 1976.
XO	♀	+	oocyte	mouse: Welshon and Russel, 1959. man: Singh and Carr, 1966.
	♂	+	spermatocyte	man: Kjessler, 1966.
XX, Sxr	♂	-*	-	mouse: Cattanach, Pollard and Hawkes, 1971.
XO, Sxr	♂	+	spermatocyte	mouse: Cattanach et al. 1971.
XXY	♂	-*	-	man: Ferguson-Smith, 1959.
				mouse: Cattanach, 1961.
				pig: Breeuwsma, 1969.
XY, Tfm	♂	+	-	mouse: Lyon and Hawkes, 1970.

* Prenatal atresia

Another interesting point from Table 4.4 is the difference between XO, Sxr and XX, Sxr mice; germ cells from the former become spermatozoa although they are immotile, while those from the latter become atretic at the spermatocyte stage. This indicates that XX germ cells cannot survive in a testicular environment; hence the absence of germ cells in Klinfelters syndrome (XXY). This also shows that although the initiation of meiosis in the germ cells (XX or XY) is under somatic influence, the genotype of the germ cells also determines whether they can survive in a particular environment (see Chapter VI).

4.5 Conclusions

The rete ovarii probably secretes a diffusible meiotic inducing substance which can initiate meiosis in both male and female germ cells. The genetic status of both the somatic and germinal cells affects the development of the gonad and its germ cells.

Chapter V: Transplantation of Rat Fetal Gonads

5.1 Introduction

One approach for investigating the sexual differentiation of mammalian gonads is to transplant gonads or gonadal primordia into a different site in an adult host, of either sex, intact or castrated. Buyse (1935), Moore and Price (1942), Greene (1943), Holyoke (1949), Torrey (1950), Ozdzenski (1971), Donovan and Peddie (1973) grafted gonads into adult hosts and observed the effect of the host's hormonal environment upon the differentiation of transplanted ovaries and testes. In general, they observed that the testes developed more or less normally and that ovaries were incapable of fully autonomous development until a well developed cortex was present. This demonstrates that the hormonal environment cannot modify the initial development of the gonads in Eutherian mammals. In Marsupials, Burns (1956) and Alcorn (1975) influenced the testicular development in the opposum and the wallaby by administering a low dose of oestradiol diproprionate.

Success in modifying gonadal development by grafting embryonic testes and ovaries together, in close contact, into adult hosts had been reported by several authors (MacIntyre, 1956; MacIntyre et al., 1959; Turner and Asakawa, 1964). In these heterosexual gonadal grafts, ovarian tubular structures containing oocytes were found (MacIntyre, 1956; Turner and Asakawa, 1964). Older ovaries, transplanted in the same manner were found to inhibit the differentiation of a younger testis (MacIntyre, et al., 1959).

Unfortunately, the results of the experiments were not clearly reported. Moreover, the authors were not aware of the importance of

the rete ovarii in early ovarian development (see Chapter 4), and therefore did not graft rat ovaries before Day 16 p.c. MacIntyre's heterosexual gonadal transplantation experiment was therefore repeated, including the rete ovarii in all the ovarian grafts. Ovaries before Day 16 p.c. can be successfully grafted under the kidney capsule of adult castrated hosts.

5.2 Material and Method

The development of the gonads in the Liverpool Hooded strain of rat was studied in fetal and neonatal gonads of varying ages. The gonads were fixed in Bouin's solution and then processed for histological examination.

The castration of adult rats and the transplantation technique was described in sections 3.4.1 and 3.4.2. When gonads were transplanted before sexual differentiation, the fetuses were sexed by air-dried chromosomal preparations from the amniotic membranes. The gonads were transplanted according to the scheme in Figure 5.1, which allowed the sex of the gonad to be determined histologically at the end of the experiment. Grafting a single gonad under the kidney capsule also served as the control experiment.

The development of the transplanted gonads was compared with that of the normal gonads.

The transplantation experiment was summarised in Table 3.5.

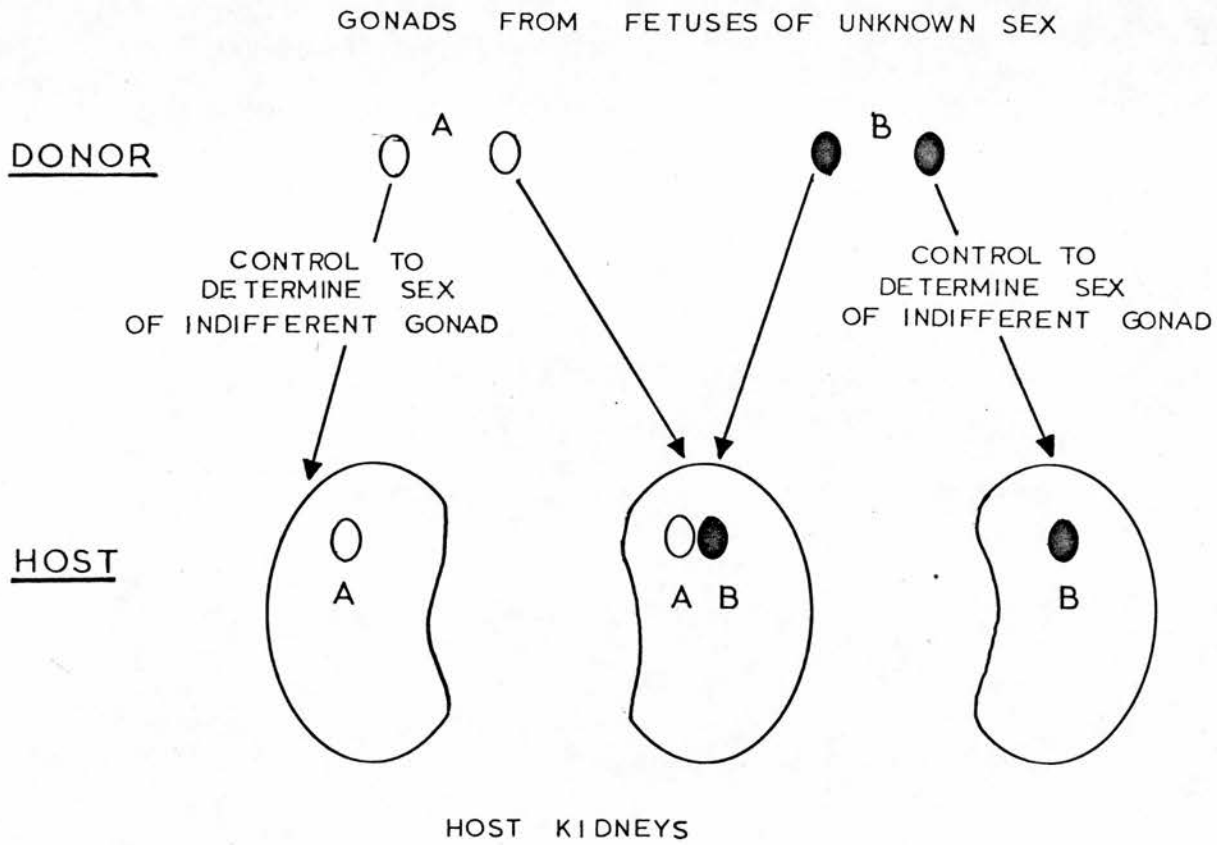


FIGURE 5.1 DIAGRAM TO SHOW METHOD OF COMBINING GONADAL ANLAGEN OF UNKNOWN SEX.

5.3 Results

5.3.1 Normal gonadal development

Gonadal differentiation was studied in the LH strain of rat fetuses. The events of sexual differentiation are summarised diagrammatically in Fig. 5.2.

Development of the germ cells was similar to that described by Clermont and Perey (1957), Beaumont and Mandl (1962, 1963) and Jost (1972). The first sign of gonadal sexual differentiation was observed in the male; this was characterised by the formation of the seminiferous cords and the differentiation of the Sertoli cells. In females, no such changes took place. The interstitial cells of Leydig and the tunica albuginea of the testis differentiated somewhat later than the seminiferous cords.

In the female, the situation was different. For a prolonged period of time, no ovarian development took place. The undifferentiated ovary just increased in size and the germ cells multiplied very actively by mitotic division. By Day 17.5 p.c. the germ cells ceased division and entered meiosis. The zygotene phase of the meiotic prophase was short (it ranged from 19.5 to 20.5 days p.c.). Diplotene germ cells could be seen on Day 1 p.p., and follicular cells could be recognised on Day 2 p.p.

5.3.2 Transplanted testis (without ovary).

Testes were transplanted alone or in pairs under the kidney capsule of adult castrated hosts as controls. Twenty-one of the

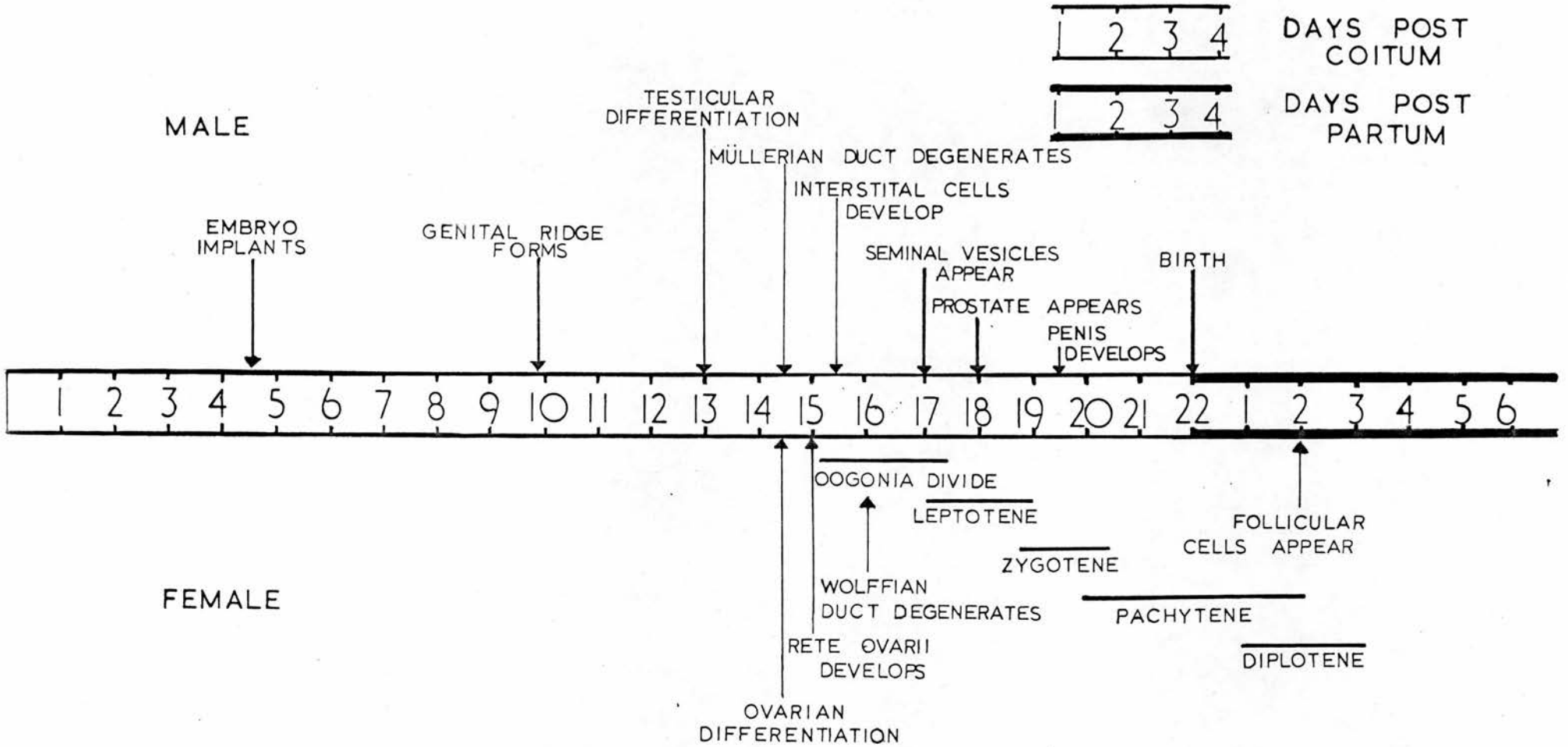


FIGURE 5.2 SEXUAL DIFFERENTIATION IN THE RAT

23 testicular grafts were recovered (91%). In all cases, testicular development was normal, with well developed seminiferous tubules and hypertrophied interstitial cells. The average seminiferous tubule diameter in the grafted testes was significantly greater ($p > 0.001$) than in the normal testis of equivalent age (Table 5.1) (Plate 2.1 and 2.2). No difference could be observed between the testicular grafts in intact and castrated hosts.

5.3.3 Transplanted ovary (without testis)

In the preliminary experiment, ovaries without rete ovarii from Day 12 and Day 14 p.c. fetuses were transplanted under the kidney capsule of castrated adult rats, but they failed to develop. After 14 days in the host, no germ cells could be found and only stromal tissue was left (Plate 2.3). The rete ovarii was included with the ovaries from Day 12 and Day 14 p.c. fetuses in all subsequent transplantations.

Of 26 control ovarian grafts with rete, 24 were recovered (92%) after 14 days. Although the early ovarian grafts did not develop as well as the later ones since they have fewer germ cells, all the oogonia completed the prophase of meiotic division, and had become primary or secondary follicles, with follicular cells. Occasionally, follicles with antra could be found in the grafts (Plate 2.4).

The rete ovarii in the ovarian grafts developed prominently (Plate 2.5), and in 5 cases, cystic structures were found in the grafts (Plate 2.6); serial sections suggested that they were derived from the rete system.

Plates 2.1 to 2.6

2.1 7 days p.p. rat testis. Note the large intertubular space and the lack of Leydig cells. H. & E., x210.

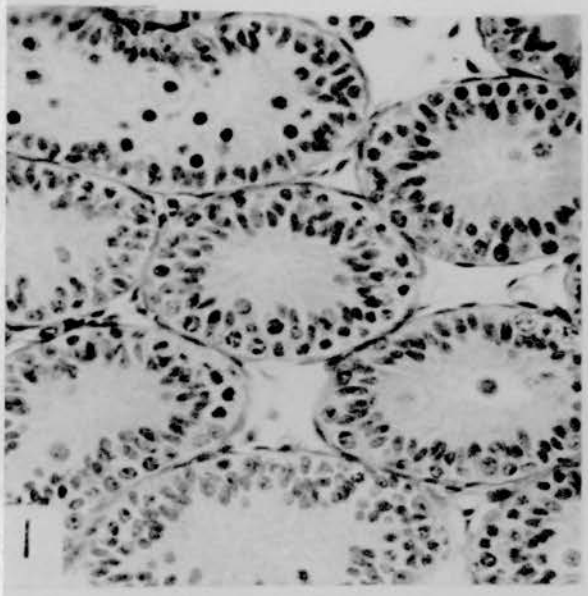
2.2 Rat testis recovered on Day 16 p.c. grafted in castrated host for 12 days (equivalent to 7 days p.p.). The seminiferous tubules are well developed but with an average diameter greater than the normal testis of equivalent age. Note the hypertrophied Leydig cells (Ly). Azan, x210.

2.3 Rat ovary recovered on Day 12 p.c. without rete ovarii and grafted in castrated host for 14 days. Only somatic cells are present in the ovarian tissue. H. & E., x350.

2.4 Rat ovary recovered on Day 14 p.c. with rete ovarii and grafted in castrated host for 14 days. Primary follicles (I) and secondary follicles (II) can be found. Azan, x 140.

2.5 Rat ovary recovered on Day 14 p.c. with rete ovarii and grafted in castrated host for 14 days. Note the hypertrophied rete system (r). The host kidney tissue (K) is also shown. Azan, x 140.

2.6 Rat ovary recovered on Day 14 p.c. with rete ovarii and grafted in castrated host for 14 days. Cyst (C) probably derived from the rete ovarii is shown. H. & E., x86.5.



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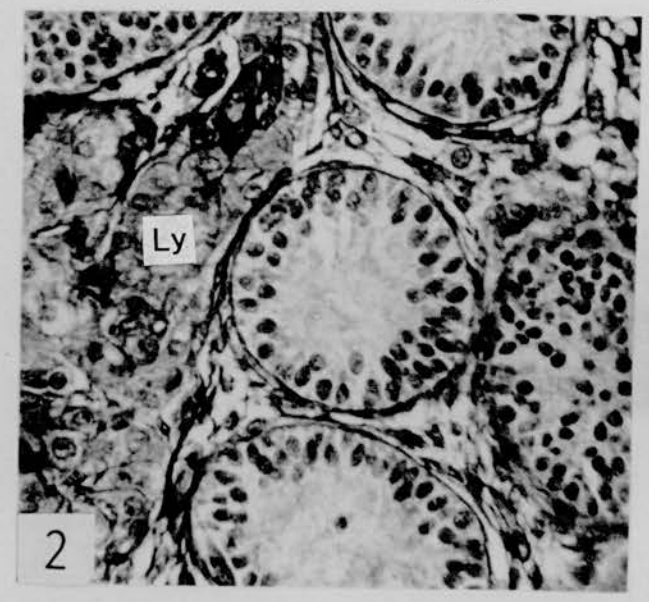

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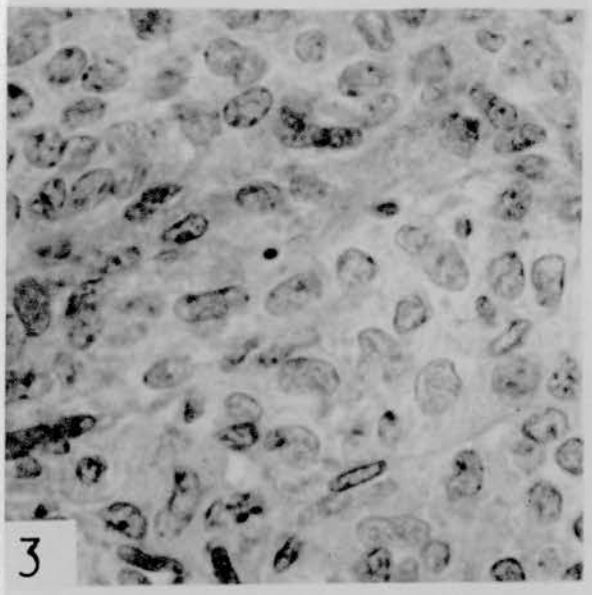

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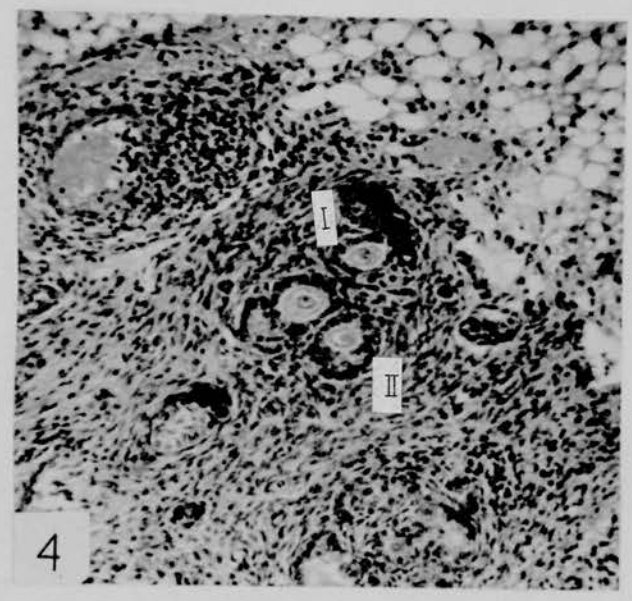

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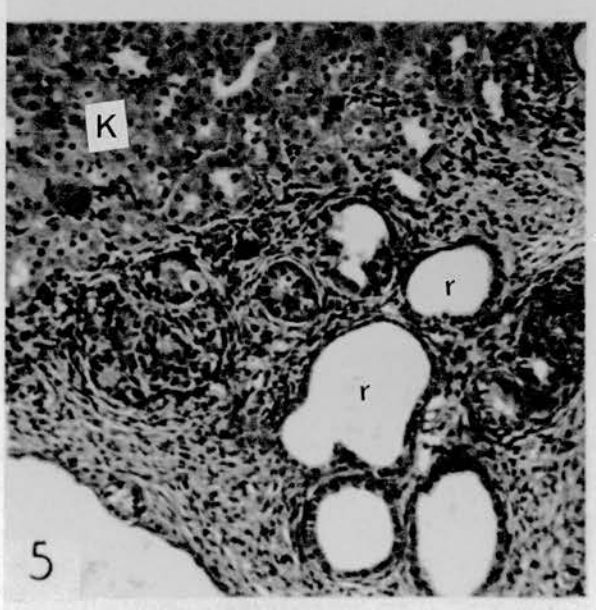

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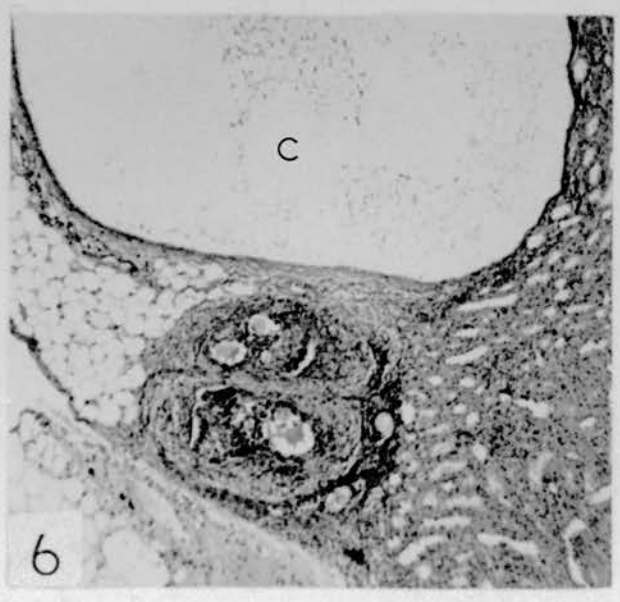

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Table 5.1: The average diameter of seminiferous tubules in the testes of normal rats and following transplantation of the fetal testis with or without an adjacent fetal ovary to the kidney capsule of an adult, castrated host.

Equivalent age of rat Days p.c.	Average diameter of tubules (μm) Developed in situ (normal controls)	Transplanted testes without ovary	Transplanted testes with ovary
5	65 ± 4	113 ± 8	110 ± 6
7	70 ± 2	115 ± 6	112 ± 8
9	73 ± 3	119 ± 10	115 ± 7
10	74 ± 4	110 ± 6	128 ± 8
11	78 ± 2	121 ± 5	119 ± 9
14	84 ± 3	136 ± 6	124 ± 7
17	108 ± 2	127 ± 4	119 ± 10

5.3.4 Heterosexual gonadal grafts

Of the 44 heterosexual gonadal grafts from various stages of gestation transplanted into adult hosts, 41 grafts were recovered (93%) 14 days after the operation. They were well vascularised and well developed.

The testicular portions of the grafts were essentially the same as the controls. Measurement of the average seminiferous tubular diameter showed that there was no significant difference between the heterosexual gonadal grafts and the controls.

Day 3 p.p. pairs and Day 20 p.c. pairs: In these grafts, both the testis and the ovaries developed normally. In the ovaries, the oogonia reached the dictyate stage of meiotic prophase and formed primary and secondary follicles. (Plate 2.7). Testicular tubules were well developed with spermatogonia inside them. (Plate 2.8).

Day 16 and Day 17 p.c. pair; The ovarian portion of the grafts contained well developed follicles with single- or multi-layered granulosa cells. Cord-like structures containing "luteinized" follicles were also observed. (Plate 2.9) In the testicular portion, the organisation of the testis was essentially the same as those in the control with well formed seminiferous tubules and hypertrophied Leydig cells.

Day 12 and Day 14 p.c. pairs: The testicular portion of the grafts developed normally as in the controls. However in the ovarian part, there was an extraordinary development of the rete when compared with the controls. In five of the grafts, atretic

follicles were observed in these rete tubules. Primary and secondary follicles could also be found in the ovarian tissue (Plate 2.9).

In the Day 14♀ + Day 16♂ pairs, testicular development appeared to be normal, while in the ovary, again atretic follicles could be found in the extensively developed rete. In the Day 14 ♂ + Day 16 ♀ pairs, both the testis and ovary developed normally, and no inhibitory effect of the ovary on the testis could be observed.

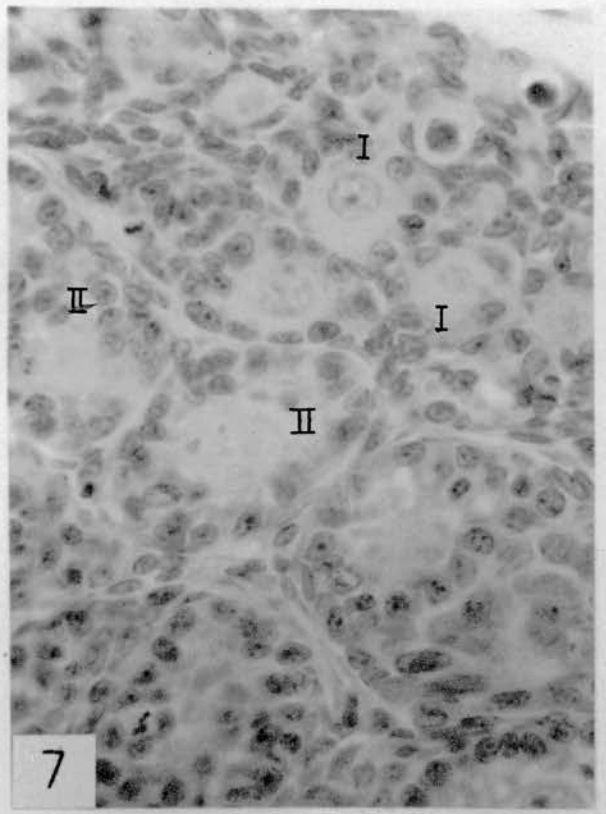
Plates 2.7 to 2.10

2.7 Rat ovary from Day 20 p.c. fetus, grafted with a testis of the same age in a castrated host for 14 days. Germ cells have completed meiotic prophase with primary (I) and secondary (II) follicles. H.&E., x210.

2.8 Rat testis from Day 20 p.c. fetus, grafted with an ovary of the same age in a castrated host for 14 days. Testicular tubules are well developed with hypertrophied Leydig cells. The kidney tissue of the host (K) is also shown. Azan, x 86.5.

2.9 Rat ovary from Day 16 p.c. fetus, grafted with a testis of the same age in a castrated host for 14 days. Normal follicles (F) can be found and 'luteinised' follicles are found in cords (arrowed). H. & E., x 86.5.

2.10 Rat ovary from Day 14 p.c. fetus, grafted with a testis of the same age in a castrated host for 14 days. Oocytes have completed meiotic prophase. The rete tubules contain atretic follicles (arrowed). Azan, x 86.5.



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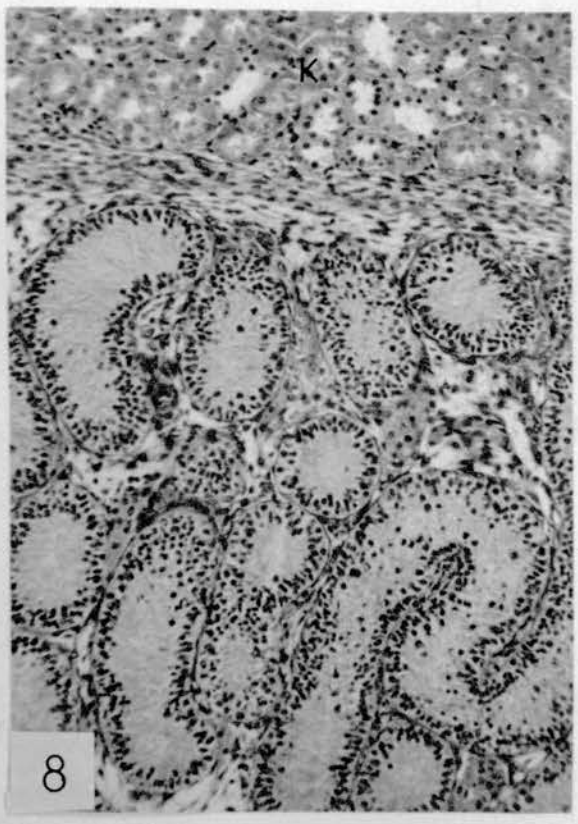

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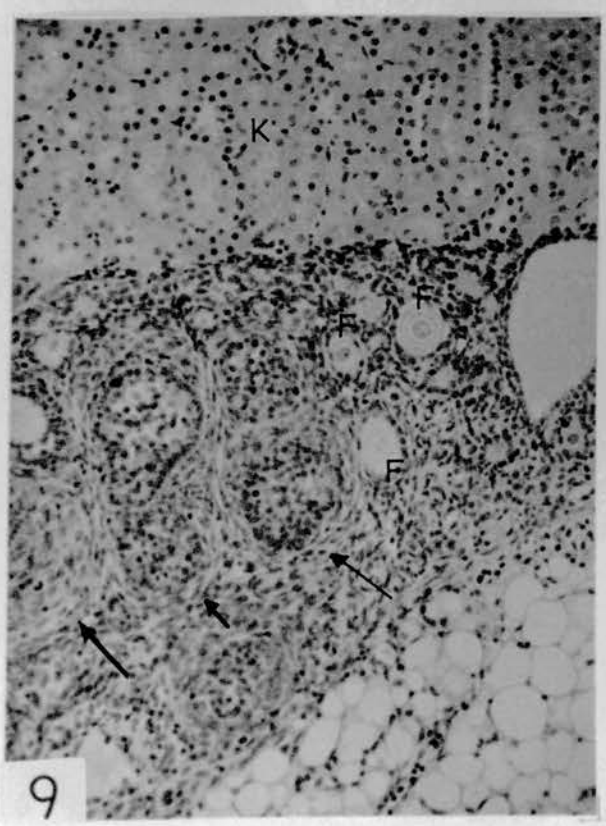

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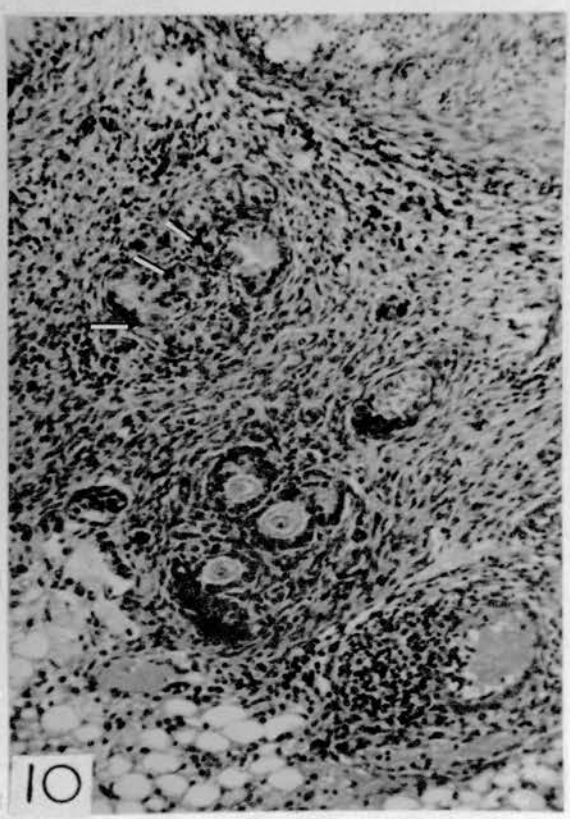

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5.3.5. Summary of transplantation results

1. Day 12 and Day 14 p.c. ovaries required the presence of the rete to develop normally when transplanted under the kidney capsule of an adult castrated or intact host of either sex.
2. A testis grafted in contact with an ovary beneath the kidney capsule of an adult host could influence the somatic organisation of ovarian development.
3. The influence of the testis on ovarian development was confined to ovaries of less than 20 days post-conceptional age.

5.4 DISCUSSION

5.4.1 Bipotentiality of the gonads

The concept that the embryonic gonads in vertebrates are developmentally bipotential has been well established. Every gonad has two discrete primordia, a medullary component which develops into a testis and a cortical component which becomes an ovary.

The bipotentiality of the gonad is best shown in fishes and amphibians where under natural or experimental conditions, spontaneous sex reversal can occur in either direction (for review, see Chan, 1970). However, among the amniotes, the bipotentiality in the embryonic gonads is not so obvious. The medullary and cortical components do not have an equal developmental potential. In birds, sex reversal usually involves masculinisation of a female and only rarely the reverse. In mammals, the freemartin conditions in cattle and some anomalous cases in human, pigs, goats are the rare naturally occurring instances of developmental expressions of medullary cords in the ovary. Experimental attempts to bring about gonadal sex reversal of any sort become more and more difficult in higher vertebrates, suggesting that the great lability shown by gonads of lower forms has, evolutionarily speaking, progressively diminished. Thus in mammals, although the structural basis of bipotentiality may still exist, the ability of the structure to respond to factors that cause morphological changes has largely been lost. The capacity of the medulla to produce testicular tissue and the cortex structure to produce ovarian tissue can be tested by setting up conditions whereby one component is given a developmental advantage over the other.

5.4.2 Experimental sex reversal of mammalian gonads

Moore and Wang (1947) dissected off the cortex from the ovaries of young animals, thus making the medullary components free from cortical influence, yet the medulla still showed some developmental capacity.

Buyse (1935) obtained ovotestes and rudimentary testes from kidney capsule grafts of rat gonads judged to be genetically female. Moore and Price (1942) found that subcutaneous gonadal grafts may convert ovaries into testes. Similarly, Mangoushi (1975) showed that scrotal grafts of rat fetal ovaries were also converted to ovotestes. Torrey (1950) found that intraocular grafts of ovaries were also transformed into testes, but the degree of development was limited. The authors reckoned that this was because of environmental inhibition in the cortical development, in contradiction to medullary inhibition of cortical development.

Ovarian germ cells of the rat and mouse have also been caused to differentiate in the male direction by isotransplanting heterosexual pairs of embryonic gonads to the kidney capsule of adult hosts (MacIntyre, 1956; MacIntyre et al., 1959; Turner and Asakawa, 1964; Mangoushi, 1977). In the present study, although seminiferous tubules were not found in the ovarian part of the heterosexual gonads, the excessive development of the rete ovarii and the retention of the cord like structure in the ovarian tissue could be considered as ovarian masculinisation. In normal testicular development, the rete testis becomes a structural part of the gonad (Roosen-Runge, 1961).

The identity of the morphogenetic factor of the young testis that is responsible for ovarian masculinisation, is still obscure. Attempts have been made to duplicate these results through administration of exogenous androgens to hosts carrying ovarian grafts, but the results were uniformly negative (Turner, 1969). This is also supported by the results by Neumann, Elger, Steinbeck and Gräf (1975) who showed that androgens take no part in sexual differentiation of the gonads. It has also been shown, in the in vitro study that initial gonadal sexual differentiation is independent of gonadotrophin (see Chapter IV), although they are essential for subsequent development. The stimulated interstitial cells and the larger average seminiferous tubule diameter in the testicular grafts, and the excessive numbers of the secondary follicles in the ovarian grafts, are presumably responses to the high gonadotrophin levels in the castrated hosts.

If the masculinisation of the ovaries is brought about by a morphogenetic factor produced by the testis, its production must have been confined to the earlier stage of development because testis after Day 20 p.c. cannot produce this effect, as shown in the present study. This morphogenetic factor may be a fetal protein, similar to the Müllerian inhibitory substance (Josso, 1972, 1975) which is only produced by the Sertoli cells before the 28th week of gestation in human pregnancies.

MacIntyre et al. (1959) claimed to have shown a general inhibitory effect of older fetal ovaries on younger testes. This is, so far, the only report of an ovarian inhibition on testicular development. This effect could not be confirmed in the present study.

The inhibitory effect described by MacIntyre et al. may have been no more than the poor ability of a young testis to survive as a graft in contrast to the improved survival of an older ovary.

5.4.3 Meiotic inductor substance

The transplantation results confirm that the rete ovarii is essential for the development of rat ovarian grafts from Day 11 to Day 14 p.c. This supports the results of the in vitro culture of hamster gonads in Chapter IV. The results are similar to those reported by Byskov (1974) who grafted mouse fetal ovaries subcutaneously into nude mice and found that the external rete ovarii was essential for ovarian differentiation before Day 14 p.c.

The induction of precocious meiosis in male germ cells was observed in the heterosexual gonadal cultures of hamster gonads in vitro (Chapter IV). No similar effect was seen in this experiment although the meiotic inductor substance secretion in the ovaries took place as shown by normal meiosis in the ovarian grafts. This difference may be due to species, or the meiotic inductor substance may not be effective in a testis exposed to high gonadotrophin levels.

5.4.4 Freemartin condition

The heterosexual gonad grafts produce an ovarian masculinisation which is closely comparable to the freemartin condition. Recently, Riveli et al. (1976) produced good evidence to suggest that some morphogenetic factors, rather than XY blood cells, from the male co-twin are responsible for the masculinisation of the freemartin ovaries.

From the developmental data on freemartin gonads (Jost et al., 1975), this morphogenetic factor must inhibit the cortical development, as shown by an arrest of gonadal growth on Day 48 p.c., and the freemartin's germ cells also do not multiply at the normal time. Meiosis of germ cells has been observed on Days 60-70 p.c. in the freemartin gonads, and after Day 90 p.c., the freemartin gonads resume their growth (Jost et al., 1975). In most freemartins, cord like structure containing germ cells could be found in fetal gonads but these cords become sterile after birth. This is similar to the present heterosexual gonadal graft results: the formation of the cords have been observed in these grafts, and they contain atretic follicles. This is in line with the belief that female (XX) germ cells are not able to survive in the male environment.

The similarity between the freemartin gonads and the heterosexual gonadal grafts brings out three interesting points in gonadal differentiation:- (1) ovarian masculinisation can apparently be brought about by some 'morphogenetic factor(s)' secreted by the testis, (2) meiosis of the female germ cells is not affected by the masculinisation of the gonad, showing that somatic organisation and the germ cells differentiation is under separate control mechanism, (3) female (XX) germ cells cannot survive in the male environment.

5.4.5 Chimaeras in mice

The production of artificial chimaeric mice by aggregating cleavage-stage of mouse embryos in vitro (Tarkowski, 1961; Mintz, 1962) made it possible to create a mammalian organism composed of

both XX and XY cells. Experiments showed that the rate of hermaphroditism was low in these XX/XY chimaeras (for review, see McLaren, 1976). In fact, most sex chimaeras become normal fertile males which had a mixed population of XY and XX cells present throughout the body (Mystkowski and Tarkoswki, 1970; Mintz, 1968). This indicates the dominant role of the male-directing morphogenetic factor in the differentiation of the gonad. This could also perhaps explain, in heterosexual gonadal grafts, ovarian masculinisation could be achieved while feminisation of testes was not observed.

This sex chimaera model cannot answer the two important issues in gonadal sex differentiation: (i) whether the phenotype of the gonad is determined by the somatic component or by the germ cells; (ii) what determines the fate of the germ cells whose genotype does not correspond to the phenotype of the animal. This is investigated and discussed in Chapter VI .

5.5 Conclusions

The rete ovarii is essential for rat fetal ovarian differentiation, confirming the results in Chapter IV. The rat fetal testis before Day 20 p.c. is capable of inducing ovarian masculinisation in fetal ovaries grafted in contact with it under the kidney capsule of an adult host. Suppression of testicular differentiation by an ovary is not observed.

Chapter VI: Chimaeric gonads

6.1 INTRODUCTION

As pointed out in the previous chapters, the identity of the actual sex determining elements within the primordial gonad is an unsolved problem. Whether it is the germ cells, somatic cells or both that determine the phenotypic sex of the gonad remains unknown. A study of aggregation XX/XY chimaeric mice (Tarkowski, 1961; Mintz, 1962) at the earliest stage at which gonadal sex can be determined perhaps throws some light on the problem.

The sexual development of these chimaeric mice has been extensively reviewed by McLaren (1976) who raised the following points:

(1) XX/XY individuals show varying degrees of dominance of the male gonadal component ranging from 100% development of testes in some strain combinations to equal numbers of testes and ovaries in others. Dominance of the XX component, with a preponderance of ovaries, has never been reported, and gonadal hermaphroditism is rare, except in the case of XX/XY^{-} Sxr chimaeric mice, where hermaphrodites are common; (Gordon, 1976).

(2) Functional sex reversal of XX germ cells has never been reported, but functional sex reversal of XY germ cells may occur (Evans, E.P., unpublished results, personal communication).

(3) What determines the phenotypic sex of the gonad remains unclear. Arguments as to whether the germ cells or the somatic components are responsible for deciding the gonadal sex have been reviewed by Tarkowski (1969, 1970), Ford (1970), Short (1972) and O and Short (1977). The drawback of using chimaeric mice for the study of gonadal sex determinations is that the cellular constitution

(XX or XY) of the adult gonads may bear little relationship to that of the genital ridge in the embryo. Therefore, breeding records of these chimaeric mice and the analysis of the adult gonads may not be very informative in solving the problem of primary gonadal development. Another limitation is that there is no way of telling the "patch size" of the XX and XY cells in the gonads.

Steinberger and Steinberger (1966) and Yamada, Yasue and Matsumoto (1972) developed techniques to separate the germ cells from the immature rat testis. Their techniques have therefore been adapted to separate germinal from somatic cells in the fetal testis and ovary. By reaggregating germ cells with somatic cells of the opposite sex, a 'chimaeric gonad' can be obtained and cultured, thereby making it possible to study the fate of germ cells in a genetically inappropriate somatic environment.

6.2 MATERIAL AND METHODS

The details for making chimaeric hamster gonads composed of XX germ cells and XY somatic cells, or vice versa, have been given in section 3.5. The method is summarised in Fig. 6.1.

The chimaeric gonads were cultured for 3 to 8 days by the method described in section 3.3.1 and at the end of culture, they were fixed for histological examination.

The culture medium used for germinal and somatic cells separation consisted of Eagle's minimal essential medium with Hank's salt buffered with HEPES and was supplemented with newborn calf serum (20% v/v), L-glutamine (2mM/L) and antibiotics (Amphotericin B, 1.5 ug/ml, Kanamycin 30ug/ml). All the media used were obtained from Flow Laboratory, Irvine, U.K.

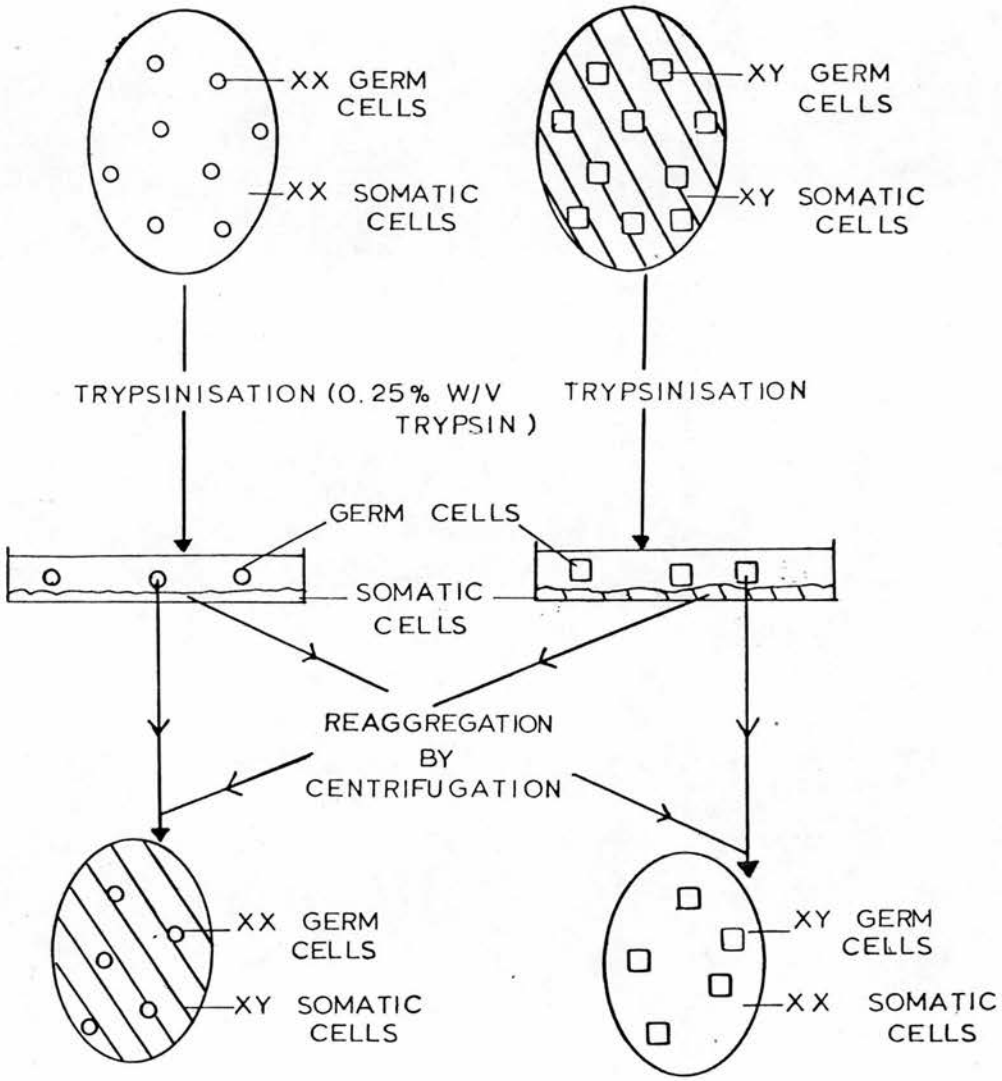


FIGURE 6.1 DIAGRAMMATIC FLOW SHEET FOR MAKING CHIMAERIC HAMSTER GONADS.

6.3 RESULTS

6.3.1 Culture of trypsinised fetal gonads

Hamster gonads were sexed using the sex chromatin method. Fetal testes and ovaries were trypsinised separately and their dissociated cells were cultured in the culture chambers. After 18 to 24 hours in culture, the somatic cells from the fetal gonads attached to the glass coverslip at the bottom of the culture chambers while the germ cells remained floating in the culture medium. It was found that the use of clean coverslips was essential if the somatic cells were to adhere to the glass.

In some cases, the glass coverslip, when removed from the chamber, was fixed in methanol-acetic acid and stained with Giemsa. Three types of testicular somatic cells could be distinguished: small elongated cells with flattened nuclei; large round cells with round or oval nuclei containing two nucleoli, and large round cells with round nuclei but containing a single nucleolus (Plate 3.1). In these monolayer cultures, no germinal cells could be found. Another interesting feature of these monolayers was that they formed a characteristic tubular pattern (see Plate 3.1), suggesting a strong tendency to form structures resembling seminiferous tubules.

Ovarian somatic cells also attached to the bottom of the culture chambers. Two types of cells could be distinguished: small cells and larger rounded cells probably derived from the pregranulosa cells (Plate 3.2) Examination of both the male and female germ cells floating in the culture medium showed that there was no obvious

Plates 3.1 to 3.4

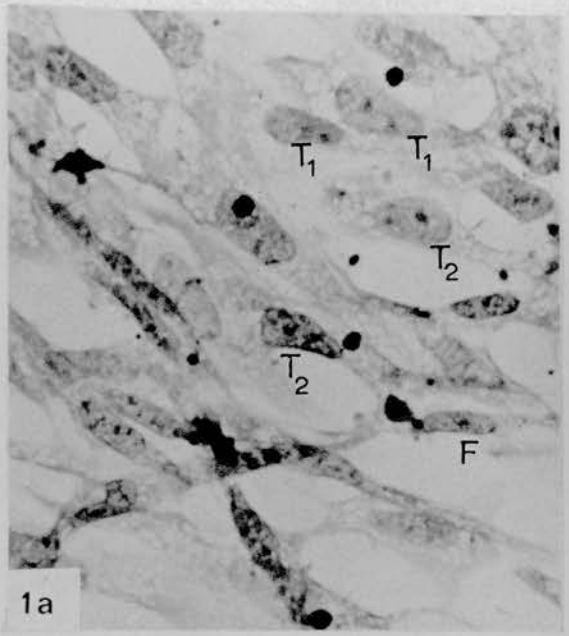
3.1a Monolayer of somatic cells from trypsinised Day 13 p.c. hamster testes after 20 hours in culture. Three different types of cells can be distinguished: fibroblastic cells (F) with elongated nuclei and two types of larger cells containing one nucleolus (T_1) or two nucleoli (T_2). Germ cells are absent. Giemsa, x 500.

3.1b Monolayer of somatic cells from trypsinised Day 13 p.c. hamster testes after 20 hours in culture. Note the characteristic pattern of growth. Giemsa, x 140.

3.2 Monolayer of somatic cells from trypsinised Day 13 p.c. hamster ovary after 20 hours in culture. No characteristic pattern of growth is shown. Two types of cells could be distinguished: the smaller fibroblastic (F) cells and the large round pregranulosa cells. Giemsa, x86.5.

3.3 XX somatic/XX germinal gonad cultured for 6 days. Most germ cells (G) entered meiosis while some become atretic (arrowed). H. & E., x 210.

3.4 XY somatic/XY germinal gonad cultured for 6 days. The germ cells (G) are dispersed amongst the somatic cells which do not reorganise into seminiferous tubules. H.& E., x500.



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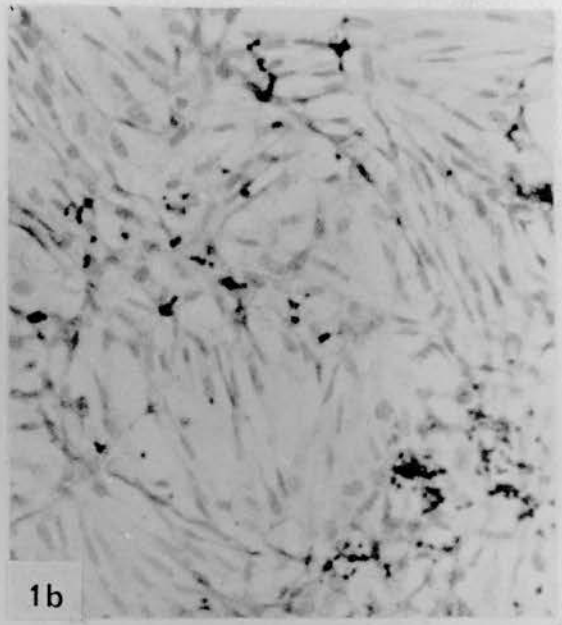

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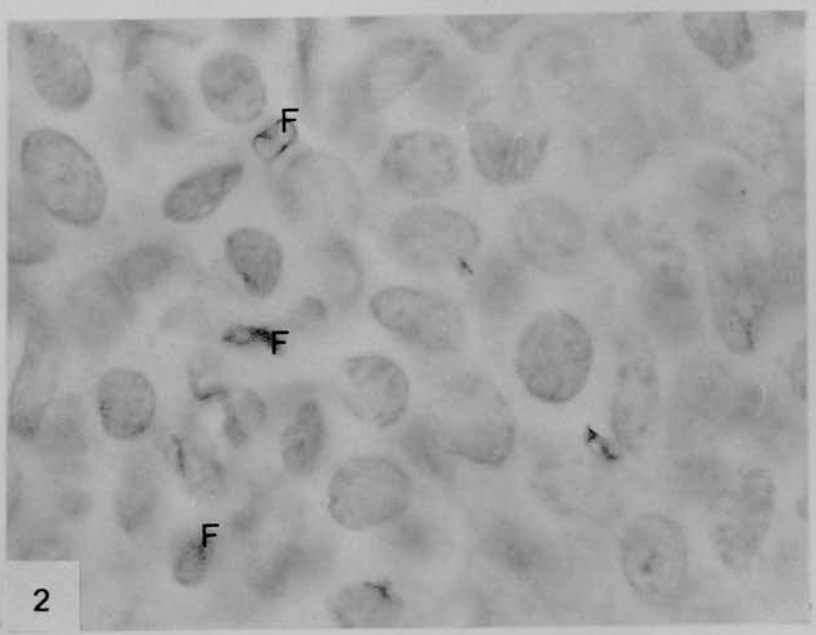

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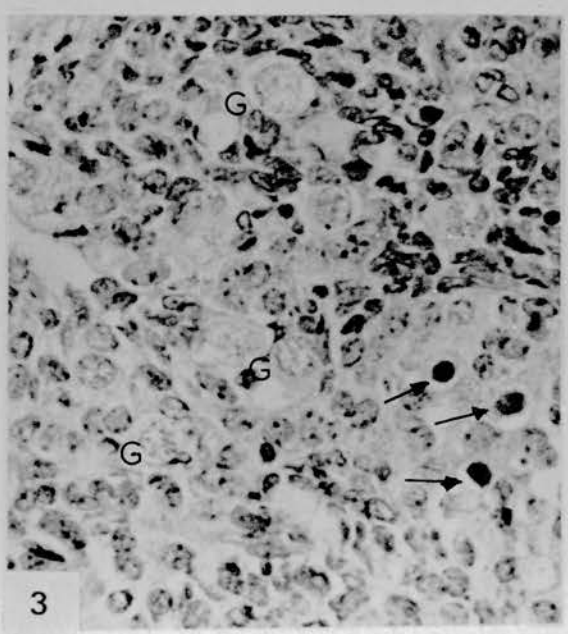

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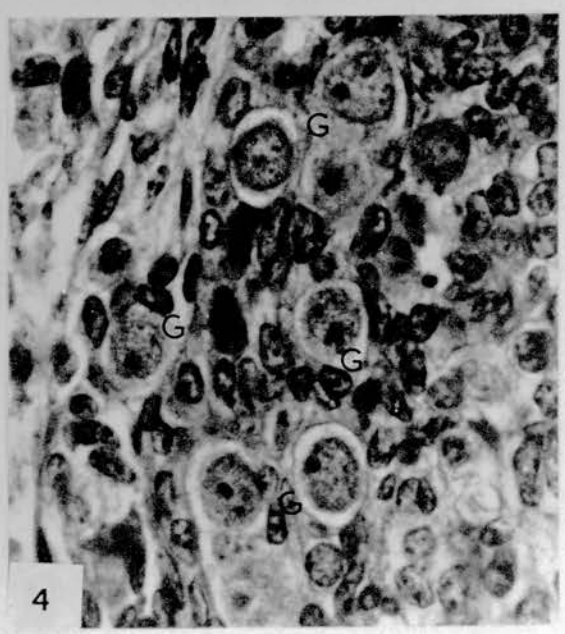

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or morphological difference between them. Furthermore, there were no differences in the dissociated cells prepared from fetal gonads collected on Day 12, 13 or 14.

6.3.2 Reaggregation of separated germinal and somatic cells

Two types of chimaeric gonads were reconstituted from the dissociated germinal and somatic cells: chimaeric hamster gonads composed of XX germ cells and XY somatic cells or vice versa. The separated germinal and somatic cells of the same genetic constitution were reaggregated together as the control. The reaggregated cells had to be kept cultured in a stationary position especially on the first two days, because the slightest shaking would disaggregate them.

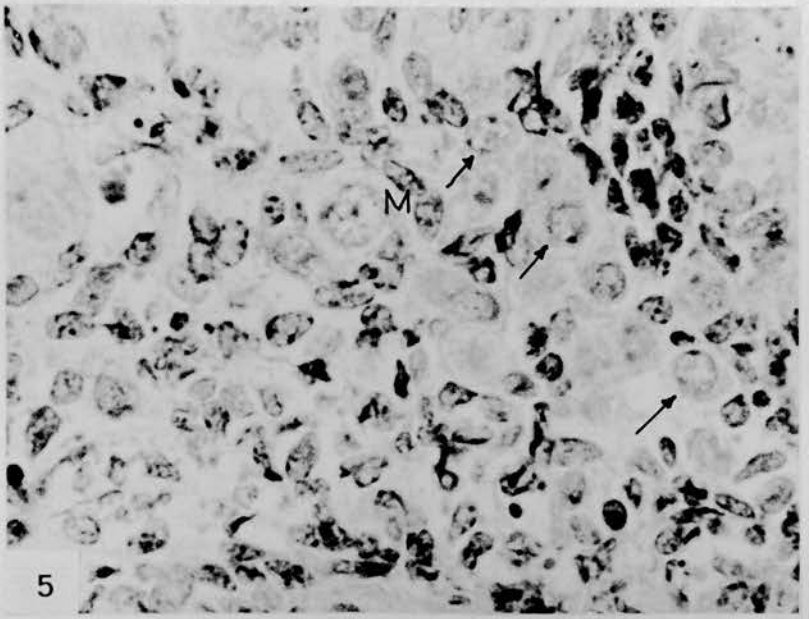
XX somatic/XX germinal gonads: Four gonads of this type were reaggregated and kept in culture for up to 8 days. The germ cells survived and most of them entered meiosis (Plate 3.3), but they remained dispersed amongst the somatic cells. The latter did not organise into cords.

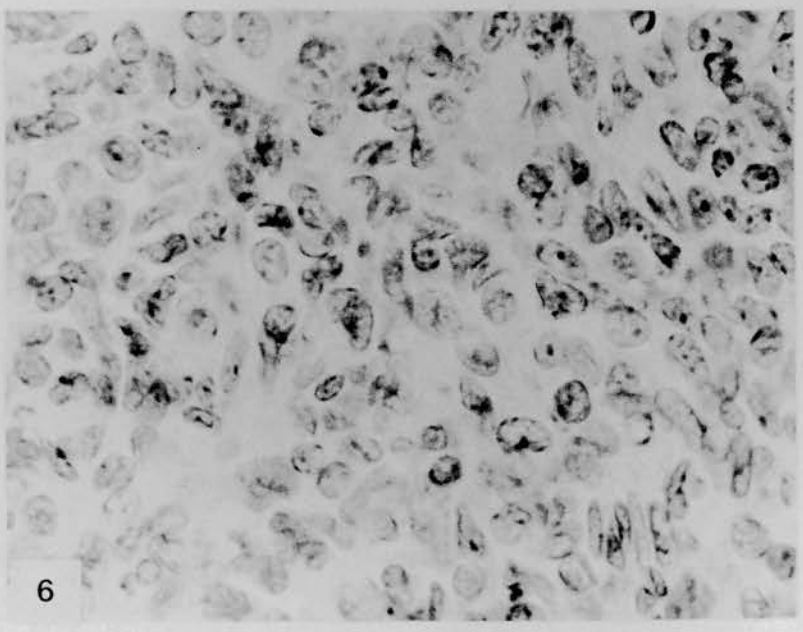
XY somatic/XY germinal gonads: Five of this type of gonads were made and in all cases the germ cells survived without entering meiosis. The somatic cells did not show the characteristic tubular pattern in reaggregation as in the monolayer culture. (Plate 3.4).

XX somatic/XY germinal gonads: Five gonads of this type were successfully made and remained aggregated after 3 to 8 days in culture. The XX somatic cells did not show any particular type of organisation with lots of fibroblastic cells in between. The XY

germinal cells became aggregated with the XX somatic cells but remained dispersed. After 6 to 8 days in culture, the nuclei of the germ cells became 'grainy' in appearance and some of them entered meiosis (Plate 3.5).

XY somatic/XX germinal gonads: Five of these chimaeric gonads were prepared and cultured for 3 to 8 days. In none of these gonads did the XX germ cells survive. Although the XY somatic cells showed a strong tendency to organise themselves into a tubular pattern in monolayer cultures, they did not appear to show the same pattern of reaggregation (Plate 3.6).





Plates 3.5 & 3.6

3.5 XX somatic/XY germinal gonad culture for 8 days. The nuclei of the XY germ cells become 'grainy' in appearance (arrowed). A germ cell (M) in meiosis is shown. H. & E., x210.

3.6. XY somatic/XX germinal gonad cultured for 6 days. No germ cells survive. H. & E., x 210.

6.4 DISCUSSION

6.4.1 Reaggregation and reorganisation of gonadal cells in vitro

Cell-cell interaction with respect to the histogenesis of the gonad and various other organs has been intensively investigated by various authors (Lilien, 1969; Morris and Moscona, 1970; Moscona, 1962, 1968; Phillips and Moscona, 1970; Steinberger, 1963). They have shown that differentiating cells in suspension have the strong tendency to reaggregate and maintain an organ-specific pattern *in vitro* which is characteristic of the cell type. In the present study, this was demonstrated in the testicular somatic cell monolayer cultures.

Lee (1968, 1971) reported that calf serum used in the medium would prevent the reaggregation of immature rat testicular cells *in vitro*, and this was thought to be caused by a non-specific effect of calf serum protein. However, such an effect of calf serum was not observed in the present study: the strong tendency of embryonic cells to aggregate may have overcome the effect.

The system of separation used in the present study depends on the characteristic large size of the germ cells and their failure to adhere to glass. The separation method is simple, and complete, and serves to separate germinal and somatic cells, enabling one subsequently to study the interactions between these cells. Lam, Furrer and Bruce (1970) demonstrated that different cell types from the rat testis could be separated by means of a difference of sedimentation velocity.

The strong tendency of male somatic cells to organise into a tubular pattern similar to that seen in the formation of seminiferous tubules is observed not only in hamster somatic gonadal cells; similar patterns have been reported in somatic monolayer cultures from fetal calf testis (Blanchard and Josso, 1975), immature rat testis (Lee, 1968) and immature chicken testis (Lee, 1971).

6.4.2 Germinal and somatic cell interaction

The present study provided a new system for investigating the interaction between germinal and somatic cells in relation to initial gonadal differentiation. In the anomalous situation of XX, Sxr mice, XX male goats and pigs and in freemartins, no XX germ cells survive in the testicular tissue, indicating that XX germ cells cannot undergo functional sex reversal. In XY/XX chimaeric male mice, Mystokowska and Tarkowski (1968) and McLaren (1975) carried out combined progeny testing and cytological analysis, and showed that only the XY germ cells line gave rise to spermatozoa. The present results have also confirmed that XX germ cells fail to survive in a somatic environment.

The fate of XY germ cells in the ovary is much more difficult to determine. So far, only two fertile female chimaeric XX/XY female mice have been reported (Ford et al., 1975). Twenty-two of the twenty-three progeny they produced were proven to be derived from the XX germ cells component of the mother. The remaining animal was XXY and the Y had come through the oocyte from the XX/XY mother. This was discussed in section 1.5.6. To what extent

XY or XXY germ cells can be transformed into oocytes when they enter an ovary remains an open question. However, there is some additional evidence to show that functional sex reversal of XY germ cells in female XX/XY chimaeras is possible. Evans (unpublished results, personal communication) recently found a single XY oocyte in meiosis in an XX/XY chimaeric female mouse. In Chapter IV, the fact that XY hamster germ cells can undergo sex reversal under the influence of the rete ovarii has been demonstrated in vitro. In the present study, the XY germ cells were found to survive and undergo precocious meiosis in the XX somatic environment and provide another line of evidence for the capability of XY germ cells to survive as oocytes.

6.5 Conclusion

The reaggregation and reorganisation of dissociated gonadal hamster cells has brought out the following points:-

(1) The fate of the germ cells is determined by both the genetic constitution and the genotype of the somatic environment.

(2) XX germ cells are not capable of surviving in the XY environment while XY germ cells can survive in the XX somatic environment and undergo precocious meiosis under the influence of these XX somatic cells.

(3) **XX germ cells are not capable of undergoing functional sex reversal while XY germ cells can be functionally sex reversed.**

Chapter VII: GENERAL DISCUSSION

In this chapter, an attempt is made to collate the literature and the findings in this thesis so as to give an overall view on our present understanding of the subject of gonadal sex determination and differentiation.

In terms of their genetic status, the mammalian male and female differ only by the presence or absence of the Y chromosome. The role of the X and the Y chromosome in gonadal differentiation has been reviewed in Chapter I (see Sections 1.3.1 and 1.3.2). There is evidence to show that Y chromosome is responsible for testicular differentiation in men (Jacobs and Ross, 1966) and in the control of testicular size in mouse (Hayward and Shire, 1973).

In eutherian mammals, there is the possibility that maternal hormones could influence the sexual development of the fetus, and maybe this is why gonadal sex determination and differentiation has become independent of hormones. However, somatic sexual differentiation is dependent on the Müllerian inhibitory substance (Josso, 1975) and testosterone (for review, see Imperato-McGinley and Peterson, 1976), while female hormones appear to play no part.

7.1 Somatic cell differentiation in the gonad

For the development of the testis, the first departure from the basic female plan is the development of the medulla. It has long been accepted that two discrete components in the gonad, the medulla and the cortex, respectively allow the gonad to develop into a testis or an ovary. However this simplistic view may be incorrect; in section 5.5.1 we saw that although mammalian gonads may be structurally bipotential, the medulla and the cortex may not have equal developmental potentials.

Chronologically, testicular development occurs earlier than ovarian development. This is clearly illustrated in the diagrams of sexual differentiation in the hamster (Fig. 4.1) and the rat (Fig. 5.1). Once the gonadal primordia have been masculinised, little possibility is left for differentiation along the female line.

Recently, immunological work (for review, see Silver and Wachtel, 1977) has suggested that there is a single testis-determining gene residing on the Y chromosome, which acts by specifying a plasma membrane protein, the H-Y antigen (for details, see section 1.3.3). Since the expression of the H-Y antigen has been claimed to be present in 50% of the 8-cell mouse embryos (Krco and Goldberg, 1976), it is probably Y-dependent rather than merely a product of testicular differentiation. However, this is a weak antigen that is difficult to detect and more confirmatory evidence is needed if we are to accept its fundamental role in gonadal differentiation.

In mammals, it is known that the testis can produce some factors that are capable of acting on the developing ovary. This was shown in the transplantation experiments (Chapter V) where testicular grafts influenced the development of an ovary grown in close proximity. The effect of the testis is to cause the retention and hyperdevelopment of the rete ovarii and Pflügers tubules, both of which contain atretic follicles. This is comparable to the masculinisation of freemartin gonads where inhibition of cortical growth and the subsequent development of seminiferous tubules occurs (e.g. Jost et al., 1973). These effects are believed to be caused by a diffusible masculinising factor which passes from the male co-twin to the freemartin via the placental vascular anastomosis, since before Day 45 p.c., ligature of the blood vessels between the

female and the male co-twin prevents the freemartin condition from occuring, although XX/XY chimaerism is evident. The identity of this masculinising factor is still unknown and requires further investigation, but Lillie's (1917) hypothesis that it is an androgen appears to be incorrect.

Although the masculinising factor from the testis inhibits cortical development and stimulates medullary differentiation in ovaries in heterosexual gonadal grafts and in freemartins, meiosis in the female germ cells is not impaired but their subsequent development is jeopardized by testicular environment. Therefore, the factor responsible for gonadal organisation is probably different from the meiotic inductor substance.

7.2 Germ cell differentiation

One major developmental difference between the male and the female is that male germ cells commence meiosis at puberty, whereas female germ cells enter meiotic prophase during fetal life. This predetermines a finite number of germ cells in the ovary early in fetal development, while large numbers of new germ cells can be produced by the testis throughout life, since 'stem' cells persist in the seminiferous tubules. This difference could be due to meiosis being actively induced in the fetal ovary, or inhibited in the prepubertal testis either actively, or because of the lack of an appropriate stimulus, or to a combination of these effects. This uncertainty has been partly resolved in this present study, since male germ cells could be made to enter meiosis precociously when hamster testes were co-cultured with ovaries. This effect

was shown to be due to the presence of a diffusible factor produced by the rete ovarii. It demonstrates firstly that male and female germ cells can enter meiosis at any time of development provided that they are given the correct stimulus, and secondly that meiosis is controlled by a factor (the meiotic inducing factor, MIS) produced by a somatic component of the gonad. By contrast, in the testis, the secretion of MIS is inhibited or postponed, presumably as a consequence of some action of the Y chromosome in the somatic cells. The initiation of meiosis in the male is under gonadotrophic control at puberty (see Fawcett, 1975), whereas only the completion of female meiosis (prophase I) appears to be hormonally controlled.

Although the prime function of germ cells in the gonad is obviously to produce gametes, the germ cells also play an important role in gonadal organisation. They may be involved in the initiation of gonadal development, since there is no evidence to suggest that gonads can differentiate in the total absence of germ cells. Bulsulphan treatment can eliminate a large number of germ cells during their migration into the primordial gonad (Merchant, 1975; Merchant-Larois, 1976), although it is not known whether any reach the gonad and initiate development before they die. Ohno et al.,(1976) have suggested that in the freemartin, XY cells from the male co-twin reach the female gonad and produce enough H-Y antigen to initiate masculinisation. However, the existence of germ cell chimaerism still has to be proved; extensive progeny-testing and meiotic studies of like-sexed cattle twins sharing a placental vascular anastomosis, and of bull calves born twin to freemartins, have failed to reveal any evidence of germ cell exchange (Ford and Evans, 1976).

The contribution of the germ cells to the structure of the gonads, especially to the ovary has been well demonstrated in this study both in ovarian cultures without the rete ovarii, and in artificially produced chimaeric gonads with XX germ cells in an XY soma. In both cases, the female germ cells died and as a result the somatic cells showed no further differentiation. In normal ovarian development, follicular cells form round the female germ cells that have reached the diplotene stage of meiotic prophase, which is Day 7 p.p. in hamsters and Day 2 p.p. in rats. By contrast, XX germ cells seemingly cannot survive in an XY environment.

The results of the organ culture study of heterosexual hamster gonads and artificial chimaeric gonads provides the first clear evidence of 'sex reversal' of XY germ cells in both an XY and an XX somatic environment. The survival of an XY oocyte is possible even with only a single dose of X chromosome and the presence of a Y chromosome. It is interesting to note that in marsupials, the genetic control of germ cell function is distinctly different from those in eutherian mammals. Robinson, Johnston and Sharman (1977) recently showed that in the tammar wallaby only one X-chromosome is functional in the oocytes. It still remains to be seen whether these XY oocytes show the characteristic germ cell vesicle and the late-labelling pattern found in meiotic prophase in XY oocytes (Kofman-Alfaro and Chandley, 1970), although this would seem unlikely. The failure of XX germ cells to survive as spermatocytes indicates that an extra 'dose' of X chromosomes jeopardises their survival (compare with the XXY, XX, Sxr and the XO/Sxr situations in Table 4.4; the XO/Sxr mice produce immotile spermatozoa, while XX/Sxr and XXY individuals are devoid of spermatocytes).

7.3 Working hypothesis of gonadal differentiation

As a working hypothesis, it is proposed that testicular differentiation can be considered as a process actively imposed upon the indifferent gonad at an early stage of development.

The indifferent gonad consists of two components of different embryonic origin: the germ cells and the somatic cells. Cortical development and the secretion of MIS from the rete ovarii is the basic plan of differentiation, which only occurs in the absence of male determining genes. Female germ cells enter meiosis under the stimulus of MIS. The meiotic germ cells interact with the somatic cells and associate closely after reaching the diplotene stage. Ovarian development before this stage is hormone independent. The female germ cells contribute to the structure as well as to the hormonal secretion of the ovary because no hormone can be produced by ovaries without follicles.

As for testicular organisation, this can only take place in an individual with a Y-chromosome (or an Sxr autosomal gene) which imposes its influence on the medulla and causes the regression of the cortex. The Y-chromosome also 'switches off' the secretion of MIS in the rete complex. The cortical development of the testis is inhibited by a masculinising factor emanating from the developing medulla. Male germ cells, which are inhibited from entering meiosis at the fetal stage owing to the lack of MIS, become enclosed in the seminiferous tubules inside which they establish a complex relationship with the Sertoli cells. This development prevents further interaction with extra-tubular cells and restricts the entry, from

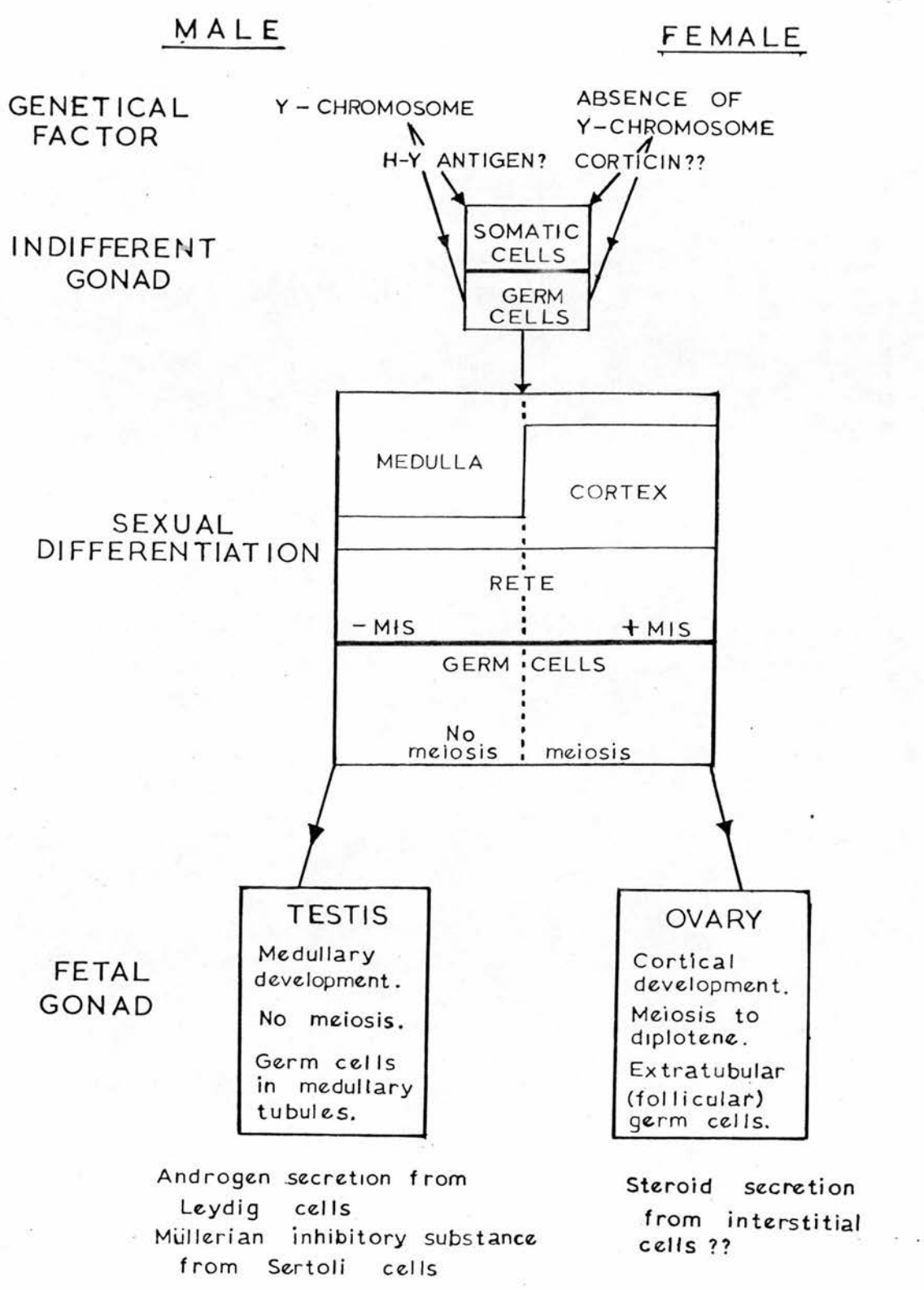


FIGURE 7.1 DIAGRAMMATIC SCHEME TO SHOW EVENTS IN NORMAL GONADAL DIFFERENTIATION.

the blood, of circulating hormones etc. The subsequent fate of the male germ cells is thus regulated by the Sertoli cells.

The scheme is represented diagrammatically in Fig. 7.1

What remains uncertain is whether it is the Y-chromosome in the germ cells or in the somatic cells that initiates male gonadal sex differentiation. The fate of the germ cells depends on their own genetic constitution, and in the case of XX germ cells also on the genotype of the soma.

7.4 Proposed future experiments on gonadal sex differentiation and determination

From the results of the present investigations, the following experiments need to be carried out as a follow-up to the present work on gonadal sex determination and differentiation:-

(1) To investigate gonadal organisation by culturing gonadal primordia before any germ cells colonise the gonadal anlagen. This work is currently in progress.

(2) To vary the culture conditions (and particularly the temperature) to test whether the inhibition of cortical development in a genetic female can sex reverse its differentiation and produce a testis as claimed by Torrey (1950) and Mangoushi (1976) in their experiments involving ovarian grafts (Pilot study commenced).

(3) To investigate the behaviour of sex reversed XY germ cells in meiosis and find out whether or not they show the characteristic sex vesicle formation and late labelling behaviour (In progress).

(4) To characterise biochemically the meiotic inductor substance secreted by the rete ovarii.

(5) Using the chimaeric gonad system, XX and XY soma can be mixed in various proportions and reaggregated to test the critical proportion of each type of cell for the control of the phenotypic development of the gonad.

(6) To transplant artificial chimaeric gonads into suitable recipients to examine the subsequent development of the gonads beyond sexual maturity.

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Initiation and control of meiosis in hamster gonads *in vitro*

Wai-sum O and T. G. Baker*

M.R.C. Unit of Reproductive Biology, 2 Forrest Road, Edinburgh EH1 2QW, and
**Department of Obstetrics & Gynaecology, University of Edinburgh, Edinburgh EH3 9ER, U.K.*

Studies of fetal ovaries maintained in organ culture have shown that meiosis can proceed *in vitro* (Martinovitch, 1938; Borghese & Venini, 1956; Baker & Neal, 1973). However, if the ovaries are removed from the fetus before the onset of meiosis, the germ cells fail to undergo normal meiosis in culture and subsequently degenerate (mouse: Wolff, 1952; sheep: Mauléon, 1973; hamster: Challoner, 1975a). In the hamster, meiosis fails to occur if the ovaries are removed before the 15th day of gestation, even if gonadotrophic hormones or maternal serum are added to the cultures, suggesting that an 'inductor' is required to initiate meiosis (Challoner, 1975a). However, the meiosis inducing factor has not been investigated further. Byskov (1974) grafted fetal ovaries into nude mice and showed that the external rete ovarii was necessary to 'trigger' the induction of meiosis in the germ cells. In the present study, the effect of the external rete ovarii on the initiation of meiosis in the hamster was studied in organ culture.

Golden hamsters (*Mesocricetus auratus*) were mated on the evening of pro-oestrus, the day following mating being designated Day 1 *post coitum* (*p.c.*). The animals were killed and fetuses were recovered on Days 12, 13, 14, 15 or 16 *p.c.*; the sex of the fetus was determined from the karyotype or from the presence of sex chromatin bodies in liver cell nuclei. The ovaries contained rete ovarii which was attached cranially to the cords of germ cells. Ovaries devoid of the extraovarian rete were obtained by trimming away the cranial part of the ovary together with the rete tubules. In the first experiment, ovaries with or without rete were maintained in culture. In the second experiment, ovaries with rete were cultured with testes recovered from fetuses of the same gestational age. As a control, testes from fetuses of similar age were cultured together. The technique of organ culture was essentially that reported by Baker & Neal (1973). At the end of the period of culture, 3–16 days within each age group, the ovaries (still attached to the agar blocks) were fixed in Bouin's fluid for 3 hr and were processed by standard dehydration and embedding procedures. Serial sections cut at 5 μm were stained with haematoxylin and eosin.

The results from both experiments are summarized in Table 1. In hamster ovaries *in vivo*, meiosis starts on Day 15 *p.c.* and the oocytes reach the diplotene stage by about Day 9 after birth (Challoner, 1974). Day 12 and Day 13 *p.c.* ovaries with external rete ovarii, maintained for 6 days in culture, contained germ cells of which more than 90% had entered meiosis (Pl. 1, Fig. 1). When ovaries with rete were cultured for 14 days most of the germ cells reached the diplotene stage of meiotic prophase (Pl. 1, Fig. 2), but the organization of the follicular cells was poor compared with normal ovarian differentiation at an equivalent age *in vivo*. Day 12 and Day 13 *p.c.* ovaries without rete cultured for 4–6 days contained only a few germ cells at the leptotene stage, and the majority did not enter meiosis. After 8 days in culture, the oogonia and the oocytes became atretic and by the 12th day the ovarian tissue was almost devoid of germ cells and consisted mainly of somatic tissue (Pl. 1, Fig. 3). Ovaries recovered with or without rete on Days 14, 15 or 16 *p.c.* contained germ cells which mainly progressed through meiosis to the diplotene stage within 12 days of culture, but once again the follicular development was poor compared with that *in vivo*. These results with ovaries cultured without rete are similar to those of Challoner (1975a) and show that the presence of the rete ovarii seems to be essential for the initiation of meiosis of hamster germ cells in culture.

In the second experiment, the gonads were cultured in close apposition in a small depression made in agar blocks. The control (testis + testis) cultures developed normally, and meiosis did not begin in the seminiferous tubules (Pl. 1, Fig. 4). In the ovary + testis cultures, ovarian development was normal, with the germ cells undergoing meiotic division up to the diplotene stage. However, in the testicular part, the tubular structure was disorganized after 4 days in culture. The somatic cells pro-

Table 1. Meiosis of fetal hamster gonads in organ culture

	Gestational age at culture (days *p.c.*)				
	12	13	14	15	16
Duration of culture (days)	6–16	5–14	4–12	3–10	3–10
Ovary with rete					
No. of cultures	4	4	5	5	3
No. of ovaries with meiosis*	4	4	5	5	3
Ovary without rete					
No. of cultures	4	4	5	5	3
No. of ovaries with meiosis*	0	0	5	5	3
Ovary with rete + testis					
No. of cultures	6	—	8	3	6
No. of ovaries with meiosis*	6	—	8	3	6
No. of testes with meiosis†	6	—	8	3	6
Testis + testis					
No. of cultures	4	—	9	9	6
No. of testes with meiosis	0	—	0	0	0

* At least 90% of surviving germ cells undergoing meiosis.
† About 30% of surviving germ cells undergoing meiosis.

liferated and surrounded the germ cells either individually or in small groups (Pl. 1, Fig. 5). These 'isolated' male germ cells entered meiosis precociously and some reached the diplotene stage of meiotic prophase (Pl. 1, Fig. 6). These results suggest that a meiosis-initiating factor, derived from the rete ovarii, can influence the development of germ cells in the testis as well as in the ovary. The possibility of migration of germinal and/or somatic cells between the ovary and testis cannot be ruled out at present but seems unlikely from the distribution of meiotic cells seen in the gonads.

These findings support the contention of Byskov (1974) that an ovarian factor, probably derived from the rete ovarii, triggers the onset of meiosis in female germ cells. Whether this depends on direct cellular contact between the rete and the germ cells or on a diffusible substance remains to be determined. Secretory activity has been reported in the rete tubules of the dog (O'Shea, 1966), cat (Mossman, 1969), and heifer (Archbald, Schultz, Fahning, Kurtz & Zemjanis, 1971). The rete cells actively secrete a PAS-positive substance at the stage when meiosis commences in the ovaries of the cat, mink and ferret (Byskov, 1975). The secretory substance has yet to be characterized, but when the fetal hamster ovary with rete attached was separated by a distance of about 2 mm from the fetal testis, testicular development was not affected and meiosis did not occur in male germ cells (W. O, unpublished observation). This indicates that if a diffusible factor is secreted by the rete, it only acts over a very short distance.

Transmission of morphogenetic signals between embryonic cells can also occur by means of intercellular contacts. It is known that cells from the spinal cord can cause the induction of mouse kidney cells. When the nervous tissue and kidney cells are separated by membrane filters, induction only occurs if cell-to-cell contact is established through the pores of the filter; no evidence for a diffusible substance was found (Wartiovaara, Nordling, Lehtonen & Saxén, 1974; Saxén, Lehtonen, Karkinen-Jääskeläinen, Nordling & Wartiovaara, 1976). Whether the rete cells and the germ cells interact by direct cytoplasmic contact remains to be investigated.

The rete ovarii is known to be influenced by hormones (man: Sauramo, 1954; heifer: Archbald *et al.*, 1971) and we cannot exclude the possibility that the rete tubules may act as mediators between systemic hormones and the germ cells, although the culture medium used in our experiments contained little gonadotrophic activity (B. M. Hobson, personal communication). This low gonadotrophic activity in the culture medium also explains the poor follicular development in the cultured ovaries as it has been shown that gonadotrophins are essential for normal follicular development in hamsters *in vitro* (Challoner, 1975b).

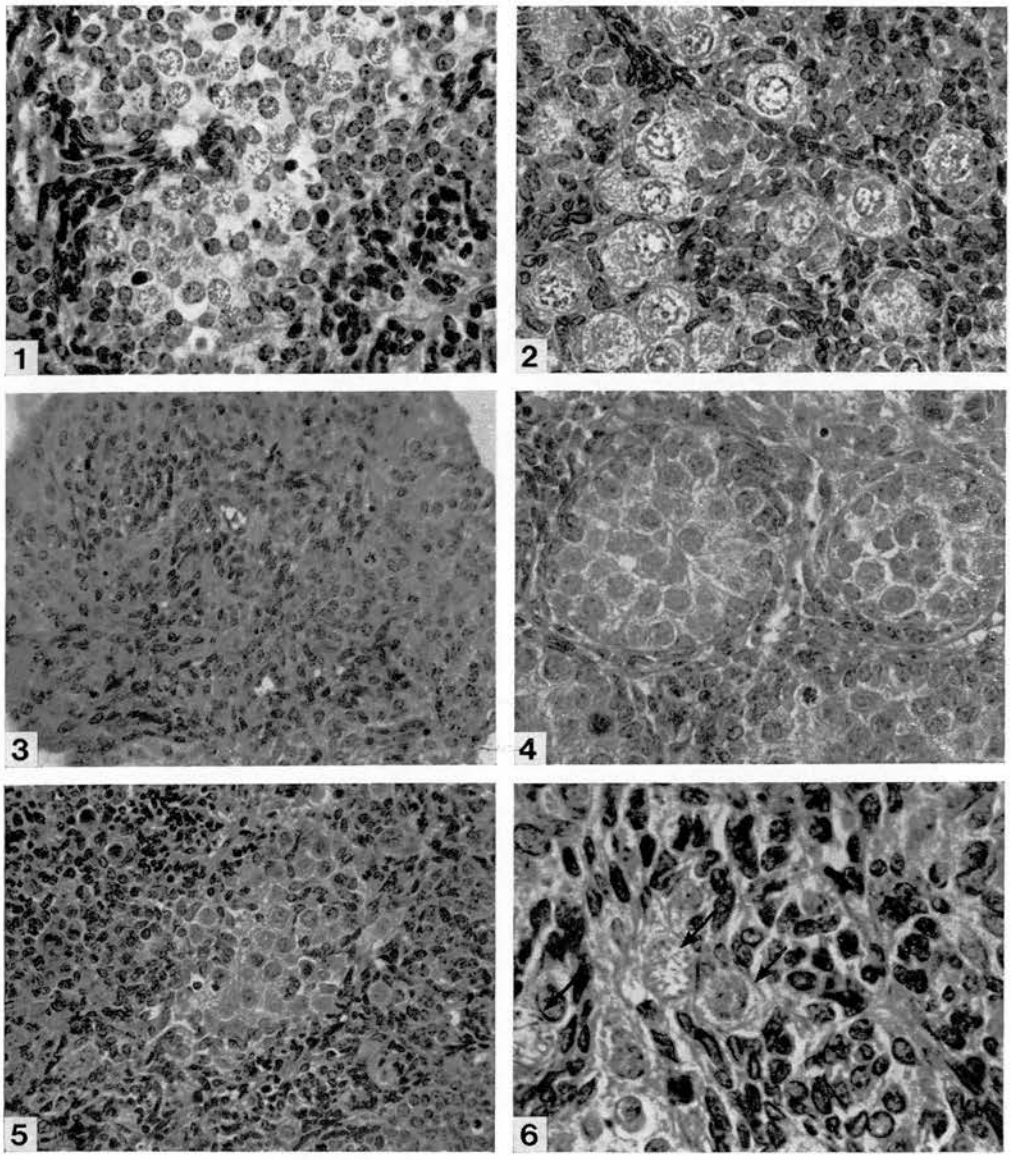

PLATE 1

Hamster gonads in culture.

Fig. 1. Ovary with rete ovarii at Day 13 *p.c.* and cultured for 7 days. The majority of oocytes are undergoing meiosis. ×580.

Fig. 2. Ovary with rete ovarii at Day 14 *p.c.* and cultured for 12 days. Oocytes have reached the diplotene stage but follicular development is severely retarded. ×580.

Fig. 3. Ovary without rete ovarii recovered on Day 12 *p.c.* and cultured for 12 days. Only somatic cells are present in the ovarian tissue. ×580.

Fig. 4. Day 14 *p.c.* testis cultured for 12 days. Seminiferous tubules and germ cells are well maintained. ×580.

Fig. 5. Day 14 *p.c.* testis cultured for 8 days with ovary of the same age, and showing disorganization of the seminiferous tubules. ×150.

Fig. 6. Day 14 *p.c.* testis cultured for 12 days with ovary of the same age. Note the large numbers of somatic cells and the male germ cells (arrowed) undergoing meiotic prophase. ×580.

(*Facing p.* 400)

Our results, showing an influence of the fetal ovary on the fetal testis, are at variance with those of earlier workers *in vitro* (rat and rabbit: Holyoke & Beber, 1958) and *in vivo* (rat: MacIntyre, 1956; mouse: Turner, 1969), who claimed to have shown an effect of the testis on ovarian development. These differences may be due to the fact that, in the present study, the hamster testis was cultured with the ovary at a time when its rete ovarii was very active. However, it is not known whether the ovarian influence is directly on the male germ cells or if the action is on male somatic cells, the initiation of meiosis being due to isolation of the male germ cells. Further studies are necessary to define more precisely this key role of the rete ovarii in the initiation of meiosis in the germ cells.

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