https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

EFFECTS OF MOUSE FETAL CONDITIONED MEDIA ON GONADAL DEVELOPMENT IN VITRO.

by

Sandra Jane Tavendale

BSc (Hons) Glasgow

Submitted for the Degree of Doctor of Philosopy

Medical Faculty, University of Glasgow

Department of Anatomy

May, 1991.

Sandra Jane Tavendale , 1991.

ProQuest Number: 11007991

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 11007991

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TABLE OF CONTENTS

	Page no.
LIST OF TABLES	3
ACKNOWLEDGEMENTS	4
SUMMARY	5
INTRODUCTION	7
MATERIALS AND METHODS	36
RESULTS	57
SECTION 1 - Testis-conditioned media	58
SECTION 2 - Ovary-conditioned media	86
SECTION 3 - Adrenal gland-conditioned media	101
SECTION 4 - Isolated germ cell cultures	115
SECTION 5 - Electrophoresis and protein analysis of conditioned media	119
SECTION 6 - Phase microscope observations	123
DISCUSSION	126
REFERENCES	154

LIST OF TABLES

TABLE 1: Extrapolation table for mouse to rat embryonic ages (dpc), extracted from Rugh (1968) - p.53.

TABLE 2: Testis-conditioned media - p.59.

TABLE 3: Quantitative analysis of cultured ovaries (±SEM) - p.65.

TABLE 4: Tunica albuginea development in cultured ovaries - p.75.

TABLE 5: Ovary-conditioned media - p.87.

TABLE 6: Proportional surface area occupied by testicular cords within 6 control 14dpc testes cultured in standard medium and 6 experimental 14dpc testes cultured in 15-17dpc ovary-CM - p.94.

TABLE 7: Adrenal gland-conditioned media - p.102.

TABLE 8: Average number of synaptonemal complexes per oocyte per ovary in 6 control 13dpc ovaries cultured in standard medium and 4 experimental 13dpc ovaries cultured in 14-17dpc adrenal-CM - 105.

ACKNOWLEDGEMENTS

I would particularly like to thank my supervisors, Dr. Sarah Mackay and Dr. R.A. Smith for their constant guidance, helpful criticism and encouragement throughout the past 3 years.

I would also like to thank Professor R.J. Scothorne for allowing me to undertake my PhD. in the Department of Anatomy. I am also grateful to the University who awarded me a Postgraduate Scholarship that enabled me to carry out this research. Thanks also go to the Queen Margaret Loan Fund for additional financial assistance.

Additional thanks go to Miss Margaret Hughes and Miss Caroline Morris for photographic work, artistic skill and their sense of humour. The technical assistance of Mr. James McGadey, Mr. Robert Kerr and Mr. Owen Reid is also greatly appreciated. Miss Brenda Robinson helped with the typing and Dr. A.P. Payne helped with statistical analysis, for which I am most grateful.

I would particularly like to thank Mrs. Heather M. Johnston for her friendship and moral support throughout the past 5 years.

Finally, I thank Dr. Philip D. Henman who acted as a constant source of encouragement and support throughout the past year, enabling me to complete this thesis on schedule.

SUMMARY

The investigation into the processes involved in fetal gonadal development has been an area of interest for many years. This thesis investigated the effects of putative diffusible factors, produced by fetal mouse ovaries and testes in culture, on normal gonadal differentiation. It was shown that testis-conditioned media from a specific age in development (13-15dpc) induced a significant reduction in germ cell number within fetal (13dpc) ovaries. Results suggested the involvement of a diffusible factor distinct from Anti-Müllerian Hormone. In addition, a significant reduction in the total surface area covered by testicular cords in section was induced by exposure of 14dpc testes to 15-17dpc ovary-CM for 4 days. Further, adrenal-CM generated from 14-17dpc produced an advance in meiosis with significantly more synaptonemal complexes than in cultured controls. A possible masculinising effect was induced by testis-conditioned media generated from 14dpc onwards, with the formation of areas of tunica albuginea resembling that of a developing testis in some ovaries. It has been suggested that male germ cells located outwith testicular cords and ectopically located within the fetal adrenal gland, enter meiotic prophase at the same time as those within equivalent ovaries (Upadhyay and Zamboni, 1982). No evidence of precocious entry into meiosis was noted in testes cultured within conditioned media generated from fetal adrenal- or

ovary-conditioned media, even when testicular cords were disrupted. In addition, isolated germ cells, cultured upon fibronectin-coated coverslips, were successfully cultured and maintained within gonadal conditioned media for up to 7 days. Electrophoretic analysis revealed proteins of a higher molecular weight in 13-15dpc testis-CM than in equivalent ovary-CM. Testis-CM generated between 17 and 19dpc, however, contained proteins of a lower molecular weight than in 17-19dpc ovary-CM indicating that distinct differences exist between developing ovaries and testes and gonads of different ages.

CHAPTER 1 : INTRODUCTION

Sexual differentiation of the gonads involves a sequence of events controlled by apparently complex mechanisms. Although extensive investigations have contributed significantly to our knowledge of sexual differentiation, especially recent genetic studies, a great deal remains to be elucidated.

As a result of a series of pioneering experiments begun in 1947, Jost formulated a theory for the fundamental mechanism of phenotypic sexual differentiation. In this, he proposed that sex differentiation involves a series of sequential processes. Genetic sex, established at fertilisation, is translated into gonadal sex which in turn directs the development of phenotypic sex of the individual.

The sexually indifferent mammalian fetus has 2 pairs of genital duct systems, one of which degenerates in each sex. The Wolffian or mesonephric duct contributes to the male reproductive tract (the epididymis, vas deferens, seminal vesicle and ejaculatory duct). The Müllerian, or paramesonephric, duct develops into the female tract - the Fallopian tube and uterine horn cranially and caudally fusing to form the uterus and part of the vagina.

Jost found that when the sexually differentiated gonads of both male and female rabbit fetuses were excised (prior to genital duct differentiation), a phenotypic female developed regardless of original genetic sex (Jost,

1947; 1953). These results showed that a testis must be present if a male is to develop, regardless of the sex chromosome constitution, ie. the basic pathway of sexual differentiation is female. The female genital ducts develop regardless of whether an ovary is present or not.

A crystal of testosterone propionate, placed near a fetal ovary *in vivo*, maintained the female Wolffian ducts fully, but did not affect the Müllerian ducts (Jost, 1947). The fetal testis produces testosterone and these results indicated that it was responsible for the maintenance of the Wolffian ducts. Later studies found that another testicular factor, termed variously Anti-Müllerian Hormone (AMH), Müllerian Inhibiting Substance (MIS) or Factor (MIF) induces regression of the Müllerian ducts (Josso *et al.*, 1977).

Gonadal development in the mouse

The presumptive gonads, or genital ridges, first become apparent as thickened areas of the coelomic epithelium with underlying mesenchyme on the medial aspects of the paired mesonephroi, at approximately 9 days post coitum (dpc).

The genital ridges undergo an identical developmental pathway for around 3 days, during which time the sex of the developing gonads cannot be determined morphologically and remains, therefore, indifferent until the onset of sexual differentiation of the testis at approximately 12.5dpc.

Prior to this period the indifferent gonads become populated with the primordial germ cells (PGCs).

Evidence suggests that the somatic cells of the developing gonads may be derived from 3 sources:- coelomic epithelium, mesenchyme and mesonephric tubules. Controversy remains, however, as to the relative contributions of each cell population. There are apparent differences between species which may be related to the state of functional activity of the mesonephros (McLaren, 1985).

Within the developing gonad cells from different sources come together and integrate to form a highly specialised structure involved in either the production of eggs in the female or spermatozoa in the male. The thickened coelomic epithelium overlies mesenchymal cells and rests on a basal lamina which, if present, is discontinuous (Byskov, 1978a; Merchant, 1975).

The coelomic epithelium was incorrectly thought to be the site of origin of the germ cells for many years and was thus known as the germinal epithelium. In certain species, with functional mesonephroi, cells of the coelomic epithelium participate in gonad formation (Yoshinaga et al., 1988; Makabe and Motta, 1986). Cells from the coelomic epithelium may infiltrate the gonadal tissue, surrounding the germ cells in both males and females, differentiating into the future Sertoli and granulosa cells respectively (Wartenberg, 1989).

The mouse gonadal blastema is thought to receive the majority of its somatic cells from the closely-associated

mesonephros, which is non-functional in this species (Byskov, 1978a; Grinsted, 1981; Upadhyay et al., 1981).

In male development the mesonephros is important in forming a connection between the seminiferous cords and the ejaculatory duct. Upadhyay and coworkers believe that the complete set of somatic cells derives from the regressing mesonephros in mouse, including the follicle and pre-Sertoli cells, which, therefore, share a common origin (Upadhyay et al., 1979; 1981). However, this is difficult to prove experimentally in the mouse due to the lack of a specific labelling system.

It is now well accepted that the PGCs arise extragonadally, although the reason for such an origin remains a matter of speculation. It may be that the comparatively late differentiation of the germ cell lineage, combined with their 'ectopic' location act to preserve their pluripotentiality. PGCs are first identifiable at around 8dpc within the mesoderm at the base of the allantois, in the caudal end of the primitive streak, and within the wall of the yolk sac (Chiquoine, 1954; Mintz and Russell, 1957; Tam and Snow, 1981). Germ cells number approximately 100 at this time.

Current evidence favours the epiblast as the ultimate source of the germ cell lineage. Diwan and Stevens (1976) showed that the epiblast layer of a 6dpc mouse egg cylinder stage embryo is pluripotent. Portions of extraembryonic ectoderm, primitive endoderm and epiblast were grafted into

the testes of adult males for a prolonged period (30 days). Only the epiblast formed a teratocarcinoma, containing cells from all 3 germ layers, including embryonal carcinoma cells which share many of the characteristics of PGCS (Hahnel and Eddy, 1987; Heath and Wylie, 1981). Snow, in 1981, found that when a small piece of the caudal end of the primitive streak was removed from a 7 days post coitum (dpc) mouse embryo and cultured in vitro for 24 hours, large round, alkaline phosphatase positive cells resembling PGCs appeared.

Chiquoine (1954) used the enzyme alkaline phosphatase in the histochemical identification of PGCs. Although not confined to the germ cell line, alkaline phosphatase has proven an accurate germ cell marker for the mouse when used in conjunction with both the characteristic morphology and location of the PGCs.

More recently, a number of alternative germ cell markers have been developed, which include monoclonal antibodies, anti-sera and radioactive labels (Heath, 1978; Heath and Wylie, 1981; Hahnel and Eddy 1986, 1987; Donovan et al., 1986; De Felici et al., 1985; Fazel et al., 1987). In addition to aiding identification of the germ cells, the development of markers specifically for cell surface components was important in studying cell membrane changes during migration and early differentiation.

A rabbit anti-serum, PG-1, was developed against mouse PGCs which recognised both plasma membrane and internal cellular components in male and female germ cells from

10.5dpc, but not somatic cells (Heath, 1978; Heath and Wylie 1981). The similarity between EC cells and PGCs was tested using antibodies known to be reactive to murine EC cells. A common reactivity with EC cells and 12.5dpc germ cells was noted which disappeared after sexual differentiation. PG-1 was found to react with all EC cell lines tested by Heath (1978), but not with their differentiated progeny. Hahnel and Eddy (1986; 1987) found that their germ cell markers were also reactive with EC cells as well as pluripotent cells of earlier (than 8dpc) embryonic stages. Again, in agreement with Heath, a change in cell surface components was noted at the time of sexual differentiation when antibody staining was lost. An additional important advantage of using monoclonal antibodies is in the identification of living cells in vitro.

The morphology of the germ cell varies slightly during its development. Generally, the PGC is a relatively large rounded or oval cell, 10-13μm in diameter when first discernable (Spiegelman and Bennett, 1973; Clark and Eddy, 1975), with cell surface extensions during the migratory phase and a correspondingly large nucleocytoplasmic ratio. The nucleus is often lobed in appearance, containing a prominent nucleolus (Jeon and Kennedy, 1973; Zamboni and Merchant, 1973). The mitochondria at 9dpc are larger than those of surrounding somatic cells (Zamboni and Merchant, 1973; Clark and Eddy, 1975). Zamboni and Merchant (1973) revealed that toluidine blue has a marked affinity for germ

cells, indicative of ribosomes.

By 9-9.5dpc, the hindgut is closed and separated from the yolk sac. Morphologically, PGCs now more closely resemble later stage germ cells, dense cored vesicles appear and mitochondria become larger and rounded. The majority of germ cells are enclosed within the gut endoderm, excluded from the gut cavity by the tight apical junctions of the lining cells (Spiegelman and Bennett, 1973; Zamboni and Merchant, 1973). A complete basal lamina underlies the epithelium of the hindgut, which was absent one day earlier and allowed free passage of germ cells between the surrounding mesoderm and the endoderm (Zamboni and Merchant, 1973).

It is generally accepted that the PGCs undergo 2 different types of migration, passive and active. Within 24 hours of their first appearance, the germ cells become passively incorporated within the endodermal lining of the hindgut due to the normal morphogenetic movements of the turning mouse embryo, as the yolk sac loses its continuity with the hindgut (8-9dpc) (McLaren, 1981).

In order to reach the genital ridge area, the PGCs must pass up the newly formed dorsal mesentery in which the gut tube is suspended (10-11dpc) and around the coelomic angles where the mesentery is attached to the dorsal body wall. They then pass laterally towards the genital ridges (11-12dpc) on either side of the midline (McLaren, 1981). This active migratory period lasts for 3-4 days by the end of which time the gonads are fully colonised. During

migration, PGCs divide mitotically increasing their numbers to approximately 4000-5000 (Mintz and Russell, 1957; Tam and Snow, 1981; Regenass *et al.*, 1982). Once within the gonadal primordia, germ cells of both sexes, continue to divide (for 2-3 days), increasing their numbers rapidly (McLaren, 1984).

In active migration, contact guidance, involving interaction with both somatic and extra-cellular matrix (ECM) components, and chemotaxis may all be involved. Jeon and Kennedy (1973) suggested that the whole mode of migration was passive, basing their hypothesis on the observation that most migratory PGCs were smooth in outline. Clark and Eddy (1975) suggested that the angle at which the sections were cut may account for this deduction.

Pseudopodial extensions of the PGCs in a cranial direction (ie. towards the genital ridge primordia) were observed by Clark and Eddy (1975), suggesting active migration within the hindgut wall for a short time prior to movement out into the dorsal mesentery. The fact that the germ cells were moving towards the gonads suggests that some direction finding mechanism was already in operation. At this time, the genital ridges have just begun to appear (9-10dpc).

In vitro studies have shown that germ cells have, at least, the ability to migrate, displaying all the characteristics of motile cells (Blandau *et al.*, 1963;

Donovan et al., 1986; Merchant- Larios and Alvarez-Buylla, 1986). This has been substantiated by others in vivo (Zamboni and Merchant, 1973; Spiegelman and Bennett, 1973; Clark and Eddy, 1975).

The extracellular environment is important in the facilitation of active germ cell migration, with surrounding cells and ECM providing substrate guidance. Orientated somatic cells may direct the germ cells towards the genital ridges, there may be an increased gradient of adhesive contacts, or an adhesive pathway.

Within the dorsal mesentery (11dpc), PGCs are associated mainly with the discontinuous basal lamina of the coelomic epithelial cells, a number are also located within the underlying mesenchyme (Zamboni and Merchant, 1973; Clark and Eddy, 1975). Leivo et al. (1980) suggested that basement membrane components are important guiding factors in morphogenetic movements of cells and tissues. Germ cells are at all times closely associated with somatic cells from which they probably obtain nutrients and developmental signals. Frequently the PGCs are either partly or completely encircled by basal cell processes of the epithelial cells (De Felici and McLaren, 1983). Those germ cells travelling within the mesenchyme are similarly enclosed by the surrounding cells. Pseudopodia extend towards the base of the mesentery (Zamboni and Merchant, 1973). Areas of close membrane contacts and interdigitations were noted between germ cells and somatic cells and, in addition, junctional complexes were observed

which apparently did not hinder active migration (Zamboni and Merchant, 1973).

Within the dorsal body wall, the germ cells are closely associated with the epithelium and mesenchyme underlying the aorta. Large intercellular spaces appear a day later, presumably as a result of hyaluronic acid production, permitting free germ cell movement through the loose mesenchyme (Zamboni and Merchant, 1973; Clark and Eddy, 1975).

In summary, PGCs are in close association with the basal aspects (basal lamina and cell processes) of the coelomic epithelial cells and also the underlying mesenchyme. In vitro and in vivo studies indicate that PGCs migrate actively out of the hindgut endoderm and towards the genital ridges.

Merchant-Larios and Alvarez-Buylla, (1986) found that mouse germ cells do not attach and translocate on glass, plastic or collagen in the absence of fibronectin, a substrate to which they can attach. Donovan et al. (1986) found that an embryo-derived cell line promoted germ cell attachment. PGCs isolated from the mesentery were actively motile and displayed invasive behaviour. A progressive decline in the ability to spread and elongate was observed during the period of genital ridge colonisation. After the period of initiation of sex differentiation of the testis, male PGCS showed reduced motility when compared to equivalent female germ cells. If substrate guidance is a

factor, a pathway of either ECM components or orientated cells would be expected. Merchant-Larios and Alvarez-Buylla (1986) used immunofluorescence techniques which revealed that fibronectin is present along the migratory route.

There is evidence for a chemotactic factor (Rogulska _et al._ 1971; Godin _et al._ (1990). When pieces of mouse embryo hindgut (containing germ cells) were grafted into the coelomic cavity of chick embryos, PGCs migrated into the chick gonads and mesonephros (Rogulska _et al._, 1971) indicating that such a chemoattractant is not species-specific. Godin and coworkers showed that 10.5dpc genital ridges attracted PGCs (isolated at the time of their first appearance) _in vitro_ and, in addition, medium conditioned by the culture of genital ridges also had a similar chemotropic efffect. Similar mechanisms have been shown to operate in other species (Kuwana _et al._, 1987 - chick; Heasman _et al._, 1981 - _Xenopus_).

Following colonisation and a period of mitotic replication, the sexes diverge and follow distinctly different pathways of differentiation.

Testicular differentiation

The first indication of sexual differentiation (as described in the rat) occurs when epithelioid cells with a clear cytoplasm, the presumptive (pre-) Sertoli cells, differentiate within the interstitial tissue near to the germ cells (Jost, 1972).

The determination of maleness in the mouse depends on the presence of Y chromosome sequences known as Tdy (testis-determining sequences on the Y chromosome). Recently a gene, Sry, located within this region has been cloned, (a gene from the sex determining region of the y chromosome). Deletion of this gene in male mice gives rise to a female. In addition, this gene was also present in Sxr' XY male mice (Sxr' is the smallest region of the mouse Y chromosome known to contain Tdy) and a mutation in this region also resulted in female development (Lovell-Badge and Robertson, 1990). The corresponding gene in man has also been identified (Sinclair et al., 1990). It was suggested that as the gene was present, albeit at a low level of expression, within the 11.5dpc testis it may act as a switch inducing differentiation of pre-Sertoli cells (Jost, 1972). These may be dependent on the cell-autonomous action of Sry. McLaren (1987a) suggested that Tdy acted at the onset of gonadal differentiation, perhaps in response to another unknown environmental factor.

Burgoyne et al. (1988) postulated that the differentiation of other testis-specific cells was controlled by the pre-Sertoli cells. It was found that while XX cells in mouse XX<->XY chimaeras contributed to the Leydig cell population, the pre-Sertoli cells were exclusively XY, suggesting cell-autonomous control of pre-Sertoli cell differentiation by Tdy. However, Hashimoto et al., (1990) suggested that Tdy is not involved in

differentiation of Sertoli cells.

Prior to the discovery of Sry, candidates for Tdy included the gene encoding H-Y antigen (a term used both for a male-specific transplantation antigen and a serologically detectable antigen), proposed by Wachtel et al. (1975) as the elusive factor, but since shown not to have a primary role in testis differentiation, as has another gene candidate, Zfy (Simpson et al., 1987). The testis-determining gene is known as TDF in humans and Tdy in mice. The region of DNA responsible for testis development has recently been mapped (Gubbay et al., 1990). Tdy is thought to act at around 11.5dpc and switches cells that would otherwise differentiate into follicle cells, to the Sertoli cell pathway.

It has been suggested that the Sertoli cells are of mesonephric origin (Upadhyay et al., 1981). The question of whether they are already differentiated as they leave the mesonephros, or whether environmental factors are involved remains unresolved.

The pre-Sertoli cells aggregate and extend processes around the germ cells leading to the formation of distinct testicular cords. Initially, the germ cells occupy a central position, surrounded by the pre-Sertoli cells (Jost, 1972). Several rounds of mitotic divisions follow before germ cells become mitotically arrested in the G1 phase of the mitotic cycle (approximately 15dpc). As testicular development proceeds, the prospermatogonia come to lie in a more peripheral position at the base of the

Sertoli cells (Hilscher et al., 1974).

Cell junctions and attachments form between pre-Sertoli cells. Peritubular cells differentiate some time later (McLaren, 1987a), outlining the testicular cord, and a basal lamina forms between the 2 cell types (Müller and Schindler, 1983). Tung et al., (1984), using in vitro techniques, suggested that epithelial-mesenchymal interactions between Sertoli cells and peritubular cells (from 20 day old rats) are involved in the formation of seminiferous tubules and production of a basement membrane. Further evidence for the importance of the ECM and epithelial-mesenchymal interactions in organogenesis was provided by Valentino et al. (1985). Addition of L-Azetidine-2-Carboxylic Acid (LACA) to cultures of mouse testes suppressed the expression of laminin and fibronectin and testicular organogenesis was prevented. Pre-Sertoli cells differentiated but failed to form cords. More recently, Hadley et al. (1990) found that laminin had a major role in Sertoli cell aggregation within a 3-D environment of reconsituted basement membrane. Polyclonal antisera inhibited cord-like formation. In contrast to Valentino et al. (1985), they found that anti-sera to collagen had no effect on aggregation. Paranko et al. (1983) showed that fibronectin is not involved in cord formation.

A tunica albuginea develops beneath the overlying flattened coelomic epithelium soon after the initiation of

testicular cord formation (15dpc). Further connective tissue organisation is evident as septa develop within the interstitium.

The prospermatogonia are now enclosed within a micro-environment of pre-Sertoli cells, which not only have a supportive role, but also provide developmental signals acting both within and without the gonad (Josso et al., 1977).

Testosterone synthesis begins at an early stage in testicular organogenesis. Taketo et al. (1984a) noted that testes removed on the 11th day gestation secreted significant amounts of testosterone after only one day's culture. Immature Leydig cells appear in vivo between 11 and 14dpc (Russo and de Rosas, 1971). Testosterone is not only involved in maintaining the Wolffian ducts, it also has a vital role in other sexually related functions.

It has been suggested that the Leydig cells differentiate under the influence of the Sertoli cells (Kitahara, 1923). Müller and Schindler (1983) hypothesised that peritubular myoid cells differentiate under the influence of testosterone.

The isolation of putative factors controlling the differentiation of Leydig cells, peritubular myoid cells and male germ cell meiosis has yet to be carried out (McLaren, 1987b).

Soon after the pre-Sertoli cells differentiate, and prior to Leydig cell differentiation, they begin to synthesise the glycoprotein Anti-Müllerian Hormone (AMH)

(Josso et al., 1977). In addition to AMH, it has been suggested that the pre-Sertoli cells produce another substance, the Meiosis Preventing Substance (MPS) (Grinsted et al., 1979).

Agelopoulou et al. and Magre and Jost reported in 1984 that, in the rat, addition of Fetal Calf Serum (FCS, 15%) to culture medium delayed the formation of testicular cords, and disrupted those already formed. Functional pre-Sertoli cells differentiated (Müllerian ducts regressed) but did not aggregate. Neither was Leydig cell differentiation and secretion affected (Patsavoudi et al.; 1985). Chartrain et al. (1984) found that only 0.5% of human serum had a similar effect. The formation of junctions between pre-Sertoli cells is apparently unnecessary for the development of normal secretory function. However, this effect seems to be species specific. In the mouse, Mackay and Smith (1989) found that addition of 10% FCS to cultures of fetal testes had no effect on the formation or development of testicular cords. Others have also added FCS to cultures of fetal mouse testes with no adverse effects reported (Hashimoto et al., 1990). Taketo et al. (1986) has suggested that FCS is actually necessary for cord formation in cultures of indifferent (11.5dpc) mouse testes, but is no longer required by those excised on 12dpc.

Meiosis and ovarian development

Within the fetal ovary the germ cells enter meiotic

prophase as primary oocytes at approximately the same time as those in the testis become mitotically quiescent (McLaren, 1981). The factor(s) involved in the control of meiosis are unknown. Both male and female germ cells must enter meiosis before further differentiation into the definitive gametes, can proceed. Although the process is basically the same in both sexes, germ cells differ in both time of entry into meiosis and its duration.

It is postulated that conditioned media may contain factors governing meiosis. In 1976, Byskov and Saxén co-cultured fetal mouse testes and ovaries and found that the normal process of meiosis could be altered, with male germ cells entering meiosis precociously and female germ cells inhibited in their development when cultured from the sexually indifferent stage. It was suggested that 2 diffusible substances acted antagonistically in both sexes to control onset of meiosis: the so-called Meiosis-Inducing Substance (MIS); and the aforementioned MPS. The mesonephros produced MIS and the Sertoli cells secreted the MPS (Byskov and Saxén, 1976; Grinsted et al., 1979; Grinsted and Byskov, 1981; Rojas et al., 1984; Grinsted and Aagesen, 1984; O and Baker, 1976). However, neither substance has been positively identified and purified, although Yding-Anderson et al. (1981) reported partial purification of MIS. Two different types of Sertoli cell have been proposed by Wartenberg (1978). 'Light' Sertoli cells (derived from the surface epithelium of the gonad)

might act directly via cell-cell contact to inhibit meiosis, whereas 'dark' Sertoli cells (from the central gonadal blastema, under the influence of the mesonephros) may be mitosis and subsequently meiosis-inducing.

The majority of authors agree that meiosis need not be induced, only prevented when within the testicular environment (Evans et al., 1977; Upadhyay and Zamboni, 1982; Taketo et al., 1984a), or when germ cells are enclosed in cords (Luciani et al., 1977; O and Baker, 1978; Rojas et al., 1984). Byskov (1978b) concluded that all germ cells that remain ectopically located within the testis or mesonephros entered meiosis. However, McLaren (1984) and Francavilla and Zamboni (1985) found that only a small proportion of male germs cells in these regions entered meiosis. O and Baker (1976) observed that meiotic male germ cells were found only in testes containing disorganised testicular cords. Ozdzenski and Presz (1984) showed that in the rat, disruption of testicular cords led to the precocious onset of meiosis. Monk and McLaren (1981) showed that X chromosome reactivation takes place at around 13dpc, prior to entry into meiosis and suggested that the signal that reactivates the X chromosome also induces entry into meiosis.

Meiotic male germ cells have also been noted within testicular cords when fluid from the epididymis of a prepubertal bull was introduced into the culture medium of fetal mouse testes (Grinsted et al. (1979). These authors

concluded that MIS was responsible.

Male germ cells differ from those of the female in the time of entry into meiosis. At approximately the same time as meiotic prophase is initiated in the ovary (14.5dpc), the germ cells within the testis become mitotically arrested and remain quiescent until a few days after birth when they pass through a stage of mitotic replication prior to entering meiosis (Hilscher et al., 1974). De Felici and McLaren (1983) found that isolated male germ cells continued mitotic divisions beyond the normal time of mitotic arrest, suggesting that the in vivo environment is necessary for initiation of this phase. The control factor(s) involved and its mode of action is unknown.

A correlation between time of entry into meiosis and cessation of excretion has been noted in several species with functional mesonephroi (Grinsted, 1981; Grinsted and Aagesen, 1984). As mentioned previously, the mesonephros was believed to secrete MIS in both males and females (Stein and Anderson, 1981; Byskov, 1978b).

A correlation with testosterone production has also been proposed in the male (Grinsted et al., 1982; Rivarola et al., 1972). Challoner (1975) found that gonadotrophins had no effect on the initiation of meiosis in the female.

In the rat, culture of similarly bisected ovaries and testes indicated that the mesonephros was unnecessary for continued development (Stein and Anderson, 1981). These authors also found that cultured sexually indifferent

gonads contained few germ cells, whereas gonads excised after morphological differentiation developed normally, but with reduced numbers of germ cells in all cultures compared to *in vivo* equivalents. In contrast to Ozdzenski and Presz (1984), no meiotic male germ cells were noted in bisected testes. Mouse fetal testes (12.5-15.5dpc), transplanted under the kidney capsule of adults contained reduced numbers of viable germ cells which had entered meiosis precociously. It was suggested that disruption of the testicular cords was directly responsible for initiation of meiosis.

The 13dpc testis contains developing testicular cords enclosing actively dividing germ cells. Studies in the rat have shown that the pre-Sertoli cells begin secretion of AMH at roughly the same time as cords form, i.e. 15dpc (Josso *et al.*, 1985).

Immature Leydig cells differentiate within the interstitium between 11 and 14dpc (Russo and de Rosas, 1971). More recently, Mackay and Smith (1989) found that Leydig cells could be identified morphologically between 15 and 17dpc in the species of mouse used in this study. Testosterone synthesis occurs within testes prior to the time of mature Leydig cell differentiation (Taketo *et al.*, 1984a). Testes removed on the 11th, 12th and 14th day gestation secreted significant amounts of testosterone following one day of culture, which increased after 2 and 3 days further culture. This is sufficient to ensure

stabilisation of the Wolffian duct. According to Dyche (1979), both pairs of genital ducts are present within the 12dpc mouse. The first sign of male Müllerian duct regression was observed on 13dpc, with the development of an epithelioid cuff. The female Wolffian duct also began to regress at 13dpc, appearing smaller than in the equivalent male and with dense amorphous substance occluding the lumen. The male and female ducts, therefore, regress in a dissimilar manner. By 18dpc, both pairs of regressing ducts are no longer present at the level of the gonads.

Anti-Müllerian Hormone

AMH synthesis commences soon after the testicular cords develop (Behringer et al., 1990). It has been shown to share sequence homology with Transforming Growth Factor β (TGFβ) and inhibin (Ueno, et al., 1989). TGFβ has both stimulatory and inhibitory effects on proliferation and differentiation of cells and, in addition, has the opposite effect to AMH on oocyte maturation (Takahashi et al., 1986).

Jost's experiments on rabbit fetuses indicated that testis differentiation and the development of the associated sex ducts, was under the control of two distinct substances, one of which was testosterone. In 1969, Picon showed that rat fetal Müllerian ducts were maintained in rat genital tracts explanted without gonads but regressed when co-cultured with fetal testicular tissue.

The gene coding AMH has since been sequenced, cloned, purified and monoclonal antibodies have been produced against it (Picard _et al._, 1986; Vigier _et al_, 1987). In addition, the bioassay, developed by Picon in 1969 has been effectively used in the detection of AMH. More recently, Donahoe _et al._ (1977) have modified this technique.

The means by which AMH exerts its regressive effect on the ducts has been determined by Price _et al._ (1977). Lysosomes increase in number, followed by a loss of polarity of duct cells which are subsequently removed by invading macrophages from the surrounding mesenchyme.

Evidence from the study of freemartins suggested that a diffusible substance, or substances, passed via the blood system from the male twin, induced masculinisation of the genetically female gonad at the time of normal morphological differentiation of the ovary (Jost _et al._, 1972).

Effects included a reduction in gonadal volume, Müllerian duct regression, germ cell depletion and, eventually, the formation of seminiferous cord-like structures. Only those gonads that contained well-differentiated cords, developed morphologically identifiable Leydig cells (Jost _et al._, 1972; 1973).

The involvement of a diffusible substance rather than the passage of cells from the male twin was shown when Vigier _et al._ (1987; 1988) reproduced the freemartin effect _in vitro_. Bovine AMH added to cultures of fetal rat

ovaries produced a reduction in oocyte number and gonadal volume along with Müllerian duct regression. It was shown that at concentrations where a reduction in germ cell number was apparent (approximately 1μg/ml-1), Müllerian duct regression was also induced, with complete regression at the level of the gonad at around 1.5μg/ml-1. Seminiferous cord-like structures developed, delineated by a continuous basement membrane.

As a result, the authors proposed that AMH is responsible for the development of freemartins and may be involved in the normal morphological differentiation of the testis. Further evidence suggests that other effects can be attributed to AMH (Josso et al., 1986). AMH is produced by the Sertoli cells of the rat, at a high concentration, until 4 days of age, even though the Müllerian ducts are totally regressed by 17dpc (in the mouse Dyche, 1979, reported regression at the level of the gonad at 18dpc in the mouse). In addition, AMH has also been detected in the ovary, specifically in the granulosa cells of mature Graafian follicles, at a time when the female Müllerian ducts are no longer sensitive to its regressive effect (when the granulosa cells differentiate). It has been suggested that AMH may act as an Oocyte Meiosis Inhibitor (OMI) (Vigier et al., 1984; Takahashi et al., 1986; Ueno et al., 1989). Further investigation into the effects of diffusible testicular factors has been carried out in heterotypic and homotypic cultures; by transplanting gonads under the kidney capsule and within the testis of adult

animals; reaggregating gonads and using medium conditioned by previous culture of testes (Byskov and Saxén, 1976; Ozdzenski et al., 1976; Burgoyne et al., 1986; Müller et al., 1982; Prépin and Hida, 1989a; 1989b).

Reduced numbers of germ cells within ovaries

The term 'germinostatic effect' was introduced by Prépin and Hida (1989b) and refers to the reduced number of germ cells in ovaries cultured under certain conditions, implying that the observed effect is specifically on germ cell mitosis. During normal ovarian development, germ cells undergo a mitotic increase in germ cell number followed by a decrease as meiosis begins (Beaumont and Mandl, 1962). Prépin et al. (1985b) showed that 13.5dpc female rat ovaries underwent an increase in the germ cell population during the first 4 days of culture, which was followed by a sharp decrease in number. Germ cell number was always lower than in equivalent in vivo ovaries. The germinostatic effect differs in that germ cells do not become pyknotic, but cease to divide during the final period of intense mitotic division (Prépin et al., 1985a).

Both fetal and postnatal testicular and postnatal ovarian diffusible factors have been shown to display germinostatic activity (Prépin et al., 1985a; Prépin et al., 1986; Charpentier and Magre, 1989; Prépin and Hida, 1989b). Vigier et al. (1987; 1988) showed that rat fetal

ovaries exposed to purified AMH do not increase their germ cell number during the period of mitotic replication prior to meiosis.

Although AMH production is common to both fetal testes and postnatal testes and ovaries, the involvement of other factors (eg. growth factors) cannot be ruled out. Indeed, separate studies (Charpentier and Magre, 1989; Prépin and Hida, 1989b) suggest that a diffusible factor distinct from AMH may produce a germinostatic effect.

Germ cells are susceptible to adverse influences prior to entry into meiosis _in vivo_ (Merchant, 1975; McLaren, 1981). Others have concluded that oocytes within meiotic prophase can be subject to degradative effects (Ozdzenski _et al._, 1976; Burgoyne _et al._, 1986).

Transplantation experiments of 12.5dpc (mouse) ovaries and testes, transplanted together but separated by a Millipore filter, under the kidney capsule of adult mice, showed that ovaries did not develop as well as when transplanted alone. Ovaries were smaller and contained few oocytes lying mainly in areas furthest away from the adjacent testis. When no filter separated pairs, the ovaries often broke down. Meiotic germ cells were, however, noted within testicular cords, which, the authors suggested, were of ovarian origin (Ozdzenski _et al._, 1976). This suggested that meiosis could continue in a male environment, even when ovarian structure was lost.

Burgoyne _et al._ (1986) also transplanted pairs of (12.5dpc) ovaries and testes under the kidney capsule of

adult female mice. Pachytene oocytes were severely depleted in numbers, or absent. Again, most oocytes present were in the regions most distant from the testis. The authors concluded, as did Ozdzenski _et al._ (1976), that the embryonic testis produced a locally diffusible factor which selectively eliminated oocytes in the prophase of meiosis. However, the time when the depletion effect occurred was not investigated.

Taketo _et al._ (1985a) found that 12 and 13dpc mouse ovaries (transplanted beneath the kidney capsule of adult males) became masculinised, with the pregranulosa cells differentiating into Sertoli cells which, it was suggested, were responsible for oocyte degeneration.

Testicular cord formation in ovaries

The involvement of a diffusible testicular factor involved in testicular cord formation has also been a subject of investigation. The differentiation of cells resembling fetal Sertoli cells in the interstitial tissue of fetal ovaries, and their aggregation into epithelial cord-like structures was noted in cultures where 16.5dpc rat testes were cultured in contact with 13.5dpc rat ovaries, and when 14.5dpc rat ovaries were cultured for a prolonged period not only in both fetal and postnatal testis-conditioned medium, but also in control medium (Charpentier and Magre, 1989; Prépin and Hida, 1989a; 1989b). Benhaim _et al._ (1982) also noted epithelial cords

(an average of 3 per ovary) in 21dpc rat ovaries cultured in control medium for 3-10 days. In addition, twice as many cords were observed in cultures containing male teratoma and lymphoma cell supernatants.

Transplantation experiments have shown contradictory results. Turner and Asakawa (1964) reported that ovaries, grafted with testes, under the kidney capsule of adult hosts, developed testicular cords. This has not been confirmed by others using similar techniques (Ozdzenski et al., 1976; Burgoyne et al., 1986). Ozdzenski and co-workers, however, noted that cord-like structures were occasionally present in areas of transplanted ovaries depleted of oocytes, but concluded that these could not be taken as a sign of masculinisation. Burgoyne et al. (1986) found that indifferent mouse ovaries had well-developed duct systems when transplanted into the adult testis which could have been taken for sterile testis tubules.

A diffusible male-specific factor inducing differentiation of testicular cells, and consequently seminiferous tubules, was proposed by Taketo et al. (1984b) who observed that the female somatic cells differentiated into Sertoli, peritubular myoid and Leydig cells. However, autonomous Sertoli cell differentiation has been suggested, as a result of Tdy expression, with other testis-specific cell types differentiating under the influence of the Sertoli cells, perhaps involving AMH (Behringer et al., 1990). Vigier et al. (1987) also found that AMH induced

Sertoli cell differentiation within cultured fetal rat ovaries. It has been suggested that the Sertoli cells induce Leydig cell differentiation (Kitahara, 1923), with peritubular myoid cells subsequently differentiating under the influence of testosterone (Müller and Schindler 1983). However, Tung et al. (1984) have suggested that interactions between the Sertoli and peritubular myoid cells were involved in testicular cord formation. This implies that peritubular myoid cells differentiate prior to the Leydig cells.

The involvement of a diffusible factor in the control of meiosis in both ovaries and testes has also been proposed (Grinsted et al., 1979).

This review has shown that there are many questions that remain to be answered in our knowledge of the processes involved in sexual differentiation. The present study shall undertake to provide further information in this area which may lead to a greater understanding of this important event in mammalian development.

The following major questions have been investigated in this thesis:-

1. Does the developing ovary secrete any controlling factors?

2. The fetal adrenal gland appears to provide a favourable environment for development of germ cells along the female pathway (Upadhyay and Zamboni, 1982). Are diffusible factors involved?

3. Do male germ cells within disorganised testicular

cords, or located outwith cords, enter meiosis?

4. Are female germ cells meiotically inhibited by a factor(s) released by fetal testes?

5. Are diffusible factors involved in the morphological differentiation of the testis?

CHAPTER 2 :MATERIALS AND METHODS

Animals and Dissection

CBA mice, from an inbred colony, were maintained on a reversed lighting regime. Males were placed with females for 4 hours, between 12 noon and 4pm, during the dark phase. Those females with a vaginal plug at the end of this period were taken to be at Day 0 of pregnancy.

At specific stages of pregnancy, animals were sacrificed by cervical dislocation. The uterine horns were dissected out and placed in a petri-dish and the fetuses, with amniotic membranes intact, were transferred into a solution of Hanks B.S.S. Buffer in order to maintain an isotonic equilibrium. All dissections were carried out in a laminar flow cabinet.

Buffer:- 9mls second distilled water
1ml 10X Hanks B.S.S. Buffer (Gibco)
0.1mls sodium bicarbonate 7.5% (Gibco)

Fetuses were placed in a Lux petri-dish containing sufficient buffer to cover the base and examined under a dissecting microscope within the laminar flow cabinet. Surrounding membranes were removed using fine needles (0.23μm, Falcon), and these were also found to be of use in further dissection steps.

The precise developmental age of each fetus was determined with reference to a method adapted from Theiler (1972), involving examination of the form of the fore and

hind limb buds (see Table 1). As developmental age can vary from chronological age by as much as 0.5 to one day, it was considered important in this study to stage fetuses immediately on removal from the mother (Mackay and Smith, 1986). Those fetuses staged at variance with those required, and any at variance with the litter as a whole were discarded.

Once staged, the upper trunk was removed, just below the fore limbs, and the abdomen was slit open in the midline. Viscera were removed and the gonads identified as paired structures lying on either side of the dorsal mesentery with the aorta visible between. The gonads were excised, together with associated mesonephroi, either in pairs or singly, from the dorsal body wall.

Prior to 12.5dpc (ie. the time of male sexual differentiation), the male and female gonads are termed 'indifferent' and are indistinguishable under the dissecting microscope. Sex was determined by staining small pieces of amnion or head ectoderm with 1% lactic acetic orcein for the presence of X-inactivated chromatin, indicative of the female genotype (Farias et al., 1967).

Following male sexual differentiation, the presence of testicular cords and distinct blood vessels allows easy identification when compared with the relatively homogeneous ovary so that sexing can be made on morphological criteria.

Culture of explants

Ovaries and testes, excised from 11.5-14dpc, were cultured in gonadal conditioned medium generated from ovaries and testes removed on 13-17dpc. Ovaries were excised both at the indifferent stage and at the time of primary male sexual differentiation and cultured in the testis-conditioned media for 2, 4 and 7 days. In addition, conditioned media were generated from ovaries excised between 13 and 15dpc, cultured for 2-3 days and collected following centrifugation and/or filtration.

Conditioned media were generated from testes excised between 13dpc and 17dpc and cultured for 2 days prior to collection following centrifugation and/or filtration.

The 13-15dpc CM-generation period was chosen as major changes are underway within the sexually differentiating testis, both morphologically and biochemically. The following series of experiments were designed to investigate the effects putative factors within 13-15dpc testis-CM have on the development of 12 and 13dpc ovaries cultured for 2-4 days. Isolated germ cells, (from testes and ovaries excised on 11.5-13dpc) were also cultured in gonadal conditioned medium generated from 14-15dpc.

An additional set of experiments involved the use of adrenal conditioned medium (generated from 14dpc pooled male and female adrenal glands cultured for 2 or 3 days) in the culture of 13dpc testes and ovaries for a period of 2 and 4 days.

a) Generation of gonadal conditioned media

Initially, the maximum number of gonads of the same sex and age obtained on each day were cultured in 3mls of standard culture medium (minus 10% FCS). CM was stored at -20°C and added to over a period of time.

Standard culture medium:- 8.8mls Williams Medium E (Gibco)
0.1mls L-Glutamine (Gibco)
0.1mls 1% gentamycin (Flow Labs.)

Testes were pre-incubated in 2.5mls or 0.8mls 0.01% Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (in Williams' Medium E) for 30 minutes at 37°C. This was to dissociate gently the developing tunica albuginea to promote the diffusion of putative factors into the culture medium.

In the majority of whole gonad experiments using gonadal CM, testes and ovaries were cultured for 2 days in 0.5mls standard culture medium. 10% FCS was omitted to allow protein analysis and electrophoresis of selected samples. Gonads were then disrupted with needles and centrifuged at 3200g for 10mins.

In some cases CM was filtered without centrifugation, in order to investigate possible retention of active factors within the media. In addition, this both sterilised media and removed any cells remaining within the CM. In one experiment, testis- CM was autoclaved to 120°C under pressure for twenty minutes to denature any proteins

present.

In Section 1.4 (the first gonad culture carried out), gonads were cultured for 1 day in 3mls standard culture medium (minus 10% FCS), disrupted as above and centrifuged for 10-20mins. Ten minutes was deemed sufficient to remove most cells. Supernatant was then filtered using a 0.22μm Millipore filter.

b) Generation of adrenal conditioned medium

Adrenal glands were obtained from mouse fetuses, removed on 14dpc, and dissected free from the cranial aspects of the developing metanephros, sex was determined according to the morphological appearance of the gonads.

Two batches of CM were generated and used in three experiments. 14dpc male (8) and female (4) adrenal glands were pooled and cultured in 2.5mls or 0.8mls of standard culture medium with added FCS for 2 or 3 days, respectively.

Conditioned media were either used immediately or were stored at -20°C. Freezing was carried out as follows:-

(i) Slow freezing - CM was placed immediately in a freezer maintained at -20°C.

(ii) Snap freezing - CM frozen within 2-3mins by liquid nitrogen cooled iso-pentane to prevent the formation of ice crystals within the CM.

Experimental and control conditions

a) Whole gonad cultures:- Pairs of gonads, with associated mesonephroi intact, were separated and one of each pair cultured in CM. The contralateral gonad acted as a culture control within standard culture medium. 10% FCS was added to both CM and control medium to support culture for periods up to a week. All procedures were carried out using sterile techniques and within a laminar flow cabinet. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. The culture period varied from 2-7 days.

b) Adrenal conditioned media:- In this set of experiments, pairs of gonads were separated and cultured in the same medium, either conditioned or control medium. FCS (10%) was already present within conditioned media, and 10% FCS was added to control cultures.

Collection of isolated germ cells

Germ cells were separated from the surrounding somatic cells using a method adapted from De Felici and McLaren (1982). As for conditioned media, gonads were treated with 0.01% EDTA prior to transfer to 0.8mls standard culture medium with added 10% FCS. Gonads were mechanically disrupted by pricking and tearing the tissue with needles and left in culture for 24 hours to allow large numbers of germ cells to pass into the medium.

Culture conditions

Halved Thermanox tissue culture coverslips were coated with 0.5mls of bovine fibronectin (10μg/ml) (Sigma) and dried at 37°C on a hotplate within a laminar flow cabinet. Coated coverslips were then sterilised by ultraviolet (UV) irradiation for one hour, and stored at -20°C until use.

Isolated PGCs (with minimal somatic cell contamination) were transferred into Nunclon multiwells containing a coated coverslip and 0.8mls of 14-15dpc testis or ovary-CM, using a sterilised, hand-pulled, glass micropipette. In some cases, 10% FCS was added. Cultures were maintained for 2-7 days at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Control cultures consisted of the residual gonadal tissue from which the isolated germ cells were obtained. They were maintained for 3-8 days and fixed at the same time as the experimental wells. Cultures were monitored using an inverted phase microscope (Leitz).

Viability testing

Viability tests were carried out on germ cells both during and at the end of culture, by means of a dye exclusion test. PGCs were micropipetted onto a dimple glass slide and a drop of 0.4% Trypan blue stain (Gibco) added. After at least 5 mins, the mean germ cell viability was calculated following examination of 3 separate reticle

fields. Viable cells excluded the blue dye, in non-viable cells dye penetrated the cell membranes (Bhuyan et al., 1976).

Fixing and processing of gonads and isolated germ cells

At the end of the culture period, gonads were fixed and embedded in Spurr's epoxy resin for examination with the light and transmission electron microscope.

In order to examine isolated germ cells in more detail, 50% of cultures upon Thermanox tissue culture coverslips were fixed and processed for further examination using the light microscope, and in some cases the scanning electron microscope.

Procedure:-

1. Fixation with 0.1M cacodylate-buffered 3% glutaraldehyde, containing 3% glucose for 30-45mins.
2. Rinse with 0.1M cacodylate buffer, containing 3% glucose for 5mins.
3. Post-fixation with 0.1M cacodylate-buffered osmium tetroxide, containing 3% glucose for 30mins.
4. Rinse with 0.1M cacodylate buffer, supplemented with 3% glucose.
5. Dehydration through a graded series of alcohols (70%, 90%, 100% x 3) for 10-15mins each.
6. Propylene oxide for 15mins.
7. Equal volumes of propylene oxide and Spurr's epoxy resin

for 30 mins.

8. Spurr's epoxy resin - overnight.

9. Fresh Spurr's epoxy resin within plastic capsules

10. Polymerised at 70°C for 48 hours

Sectioning of resin-embedded material

a) Semi-thin (approximately 1μm) sections were cut, using glass knives on Reichert-Jung Ultracut and Ultracut E microtomes, for examination using the light microscope. Sections were baked onto glass slides upon a hotplate for at least 20-30mins. Slides were stained with toluidine blue solution and rinsed after approximately 1 min. Selected slides were photographed using a Leitz Vario-Orthomat.

b) Isolated cells were processed upon the fibronectin-coated coverslips which could be cut into 4 pieces and resin-embedded in plastic capsules.

Scanning electron microscope preparation

Coverslips with attached cells were processed as for the light microscope until the last change of 100% alcohol when they were critical point dried from liquid carbon dioxide in Polaron equipment. Processed coverslips were attached to aluminium stubs with double-sided tape and coated with gold-palladium in a Polaron sputter coater. Specimens were examined in a Jeol- T-300 scanning electron

microscope.

Histochemical methods

Alkaline phosphatase histochemical identification of PGCs

The Barka and Anderson Azo-dye method (1962) was adapted and used to stain for alkaline phosphatase in both isolated germ cells and control cultures.

The substrate used was napthol AS-B1 phosphate (pH 8.5). 5mg were dissolved in 1ml dimethylformamide (DMF) and 15mls of veronal buffer (pH 9.2) added. To this solution, a few drops of an equal parts mixture of solutions A and B (solution A = 1.62g pararasanilin HCl in 50mls 2N HCL; solution B = 1.035g sodium nitrate in 50ml water) were added to produce an amber-coloured precipitate.

A few drops of solution were added to cells placed on a dimple slide, fixed by gentle flaming of the underside. Alternatively, control residual tissue and germ cells were stained within the Nunclon multiwells (placed on a hotplate maintained at 37°C) following removal of the culture medium.

Slides were immersed in reagent within petri-dishes which were placed on a hotplate (37°C) for 1-2 hours, until the characteristic red-stained germ cells were visible. Large numbers of germ cells were still present within the residual tissue by the end of culture.

The McGadey Tetrazolium method for alkaline phosphatase (1970) was also used in identification of germ

cells. 2-5mg of 5-Bromo-4-chloro-3-indolyl phosphate were dissolved in 1ml of DMF. 10mls of veronal buffer (pH 9.2) were then added along with 5mg of nitroblue tetrazolium.

Germ cells upon glass slides were immersed in reagent, as with the Barka and Anderson method, and left to incubate for 1-2 hours. At the end of this period, blue-stained cells were indicative of alkaline phosphatase positive germ cells.

Quantitative analysis

Eleven experimental ovaries, cultured in 13-15dpc testis-conditioned medium for 4 days, were completely serially sectioned at approximately 1μm thickness, together with 10 control ovaries. In addition 2 ovaries, cultured in heat-inactivated 13-15dpc testis-CM, were treated similarly. Every 20th section was retained and stained as above. Each section was photographed, using a Leitz Vario-Orthomat, at X100 and X250 magnifications. Occasional ultra-thin sections (50-70nm) were taken and stained for examination on the transmission electron microscope.

Total gonadal volume and the total number of oocytes was calculated for each ovary, using the Bioquant 3D System IV (R & M Biometrics, Inc.). The average diameter of oocytes was calculated as 24μm, following measurement of the largest oocytes per serial section in one control ovary using the same system (60 oocytes were measured). There was, therefore, a slight but consistent overestimation in

both control and experimental cultures, and therefore no correction factor was introduced. Student's t-test was used to compare the gonadal volumes and germ cell numbers of experimental and control groups.

The proportion of the total surface area covered by testicular cords was also determined using the Bioquant 3D System IV in 6 experimental testes (removed on 14dpc) cultured in 15-17dpc ovary-CM for 4 days, and 6 controls. Testes were sectioned at 1μm thickness and the surface area of the testis and the testicular cords determined in each of five sections (every 15th section retained) taken from every experimental and control testis. The proportion of the total surface area of each section occupied by testicular cords was thus determined and the results were subjected to statistical analysis (Student's t-test).

The number of synaptonemal complexes in experimental 13dpc ovaries, cultured 4 days in 14-17dpc adrenal-CM, and control ovaries was also determined. A total of 34 oocytes were examined in 6 control ovaries and in 4 experimental ovaries using the transmission electron microscope. The average number of synaptonemal complexes (within oocytes) per ovary was thus calculated and the results analysed using Student's t-test.

Biochemical analysis

a) Protein analysis

Protein analysis was carried out using a method

adapted from that of Mejbaum-Katzenellenbogen and Dobryszycka (1959).

1ml of tannin reagent (heated to 30°C) was added to each sample of the CM (made up to 1ml with Williams Medium E and distilled water). Tubes were then shaken vigorously and incubated in a 30°C waterbath for 10mins. The reaction was terminated at the end of this time by the addition of 1ml 0.2% gum acacia. Test tubes were vortexed to ensure thorough mixing of the solutions.

Tannin reagent:- 98mls 1M HCl
20g Tannic acid
4g Phenol
- heated to 80°C, cooled and filtered

A standard curve was determined using a 35% solution of bovine serum albumin.

The optical density of the BSA standard curve and the CM samples was determined using a PYE Unicam SP8-500 UV/VIS spectrophotometer, with the wavelength set at 500nm.

b) Electrophoresis

4μl samples of testis- and ovary-CM were analysed by Native polyacrylaminde gel electrophoresis using the Pharmacia Phastsystem. Two different gradient gels were used - PhastGel Gradient 8-25 and 10-15. These gels are designed to give a linear relationship between a protein's migration distance and the log. of its molecular weight for the range 50 000 to 750 000 and 90 000 to 700 000,

respectively, for native globular proteins. Commercial controls of known molecular weight were tested simultaneously. These were as follows:-

Thyroxine	-	669K
Ferritin	-	440K
Catalase	-	232K
Lactate dehydrogenase	-	140K
Albumin	-	67K

Staining procedures

Two types of staining procedures were used. Initially, gels were stained with the Fast Coomassie stain, with a sensitivity range (20-30ng of each protein/μl) and subsequently stained with silver (sensitivity range 1-5ng).

Gels were immediately placed in the development chamber of the Phastsystem and incubated consecutively in a series of solutions, according to the Development Techniques file of the Phastsystem Users Manual. Briefly, following fixation in Trichloroacetic acid (20%), proteins were sensitised by 5% glutaraldehyde following 3 successive washes in solutions of 50% ethanol and 5% acetic acid, and 10% ethanol and 5% acetic acid. Silver nitrate (0.4%) then stained proteins within separated bands after 4 consecutive washes in 10% ethanol, 5% acetic acid and reagent grade water. Gels were developed in sodium carbonate (2.5%) with added formaldehyde (2%) and the background was subsequently reduced in a solution of Tris.HCl and sodium thiosulphate.

Stained gels were photographed and dried for storage.

Criteria used in analysis of cultured ovaries

Between 10.5 and 12dpc, the male and female gonads cannot be distinguished from each other, and are therefore termed 'indifferent'. They consist of actively mitosing germ cells scattered throughout somatic tissue, which is homogeneous in appearance. The fetal gonads in the mouse remain indifferent until the time of primary sexual differentiation of the male when testicular cord formation begins (12.5-13dpc). A distinct peripheral blood vessel (extending from the renal artery), absent from equivalent ovaries, also allows easy identification of testes at this early stage in development (Fig. 1).

The testis:- Early testicular cords (13dpc) consist of peripheral, irregularly shaped pre-Sertoli cells enclosing central large, pale and rounded germ cells, which continue to divide mitotically until approximately 15dpc when a period of mitotic quiescence begins (Fig. 2) (Hilscher *et al*, 1974).

The mesonephric tubules are composed of dark-staining (using toluidene blue) cuboidal epithelial cells and are easily distinguishable from developing testicular cords (Fig. 3).

Approximately 2 days after the first testicular cords appear, a tunica albuginea develops (15dpc), consisting of 3-4 layers of flattened fibroblasts and collagen fibres covering the entire surface of the testis beneath the

flattened surface epithelium (Fig. 4). A tunica albuginea was positively identified in cultures (of either sex) only when all these features were observed. Connective tissue development is also evident between cords. Leydig cells differentiate within the vascular interstitium between 15 and 17dpc. Flattened peritubular cells outline the cords soon after their formation and the tunica albuginea is well-developed (Fig. 5).

<u>The ovary</u>:- The 13dpc ovary consists of clusters of mitotically dividing germ cells becoming arranged within indistinct ovigerous cords, outlined by stromal cells and capillaries (Fig. 6). (In this study, the ovigerous cord is taken to be a group of germ cells surrounded by flattened somatic cells.) At approximately 14.5dpc the first germ cells enter meiotic prophase as primary oocytes (Odor and Blandau, 1969).

On 15dpc (Fig. 7), flattened pre-follicle cells surround groups of germ cells as the outline of ovigerous cords becomes more defined. Between 15 and 17dpc, pre-follicular cell processes extend between adjacent germ cells. Ovigerous cords are distinct by 17dpc and electron microscopic examination reveals synaptonemal complexes within oocyte nuclei indicating that meiosis has proceeded (Fig. 8 and inset). Individual follicles begin to form at this stage (Mitchell and Burghardt, 1986).

Organisation of ovarian structure takes place from 16dpc as connective tissue septa and blood vessels

penetrate the tissue (Upadhyay *et al.*, 1979).

Duct development:- The Wolffian duct is present before 10dpc when the genital ridges begin to develop on the ventral aspects of the paired mesonephroi. Müllerian duct formation begins at around 12dpc, after a definitive genital ridge is present. Dyche (1979) noted that both pairs of ducts were already present in the 12dpc gonad with signs of regression apparent one day later. However, a different method for determining the onset of the gestation period was used and this may account for the apparent discrepancy.

A comparison of the developmental stages of mice and rats is given in Table 1. It should be noted that this can only act as a general guide as the strain of animals used by Rugh was not given.

Table 1

General extrapolation table for mouse to rat embryonic ages (dpc) (extracted from Rugh, 1968)

Mouse	Rat
10	11.5
10.5	12.125
11	12.5
12	13
12.5	13.5
13	14.5
14.5	15.5
15	16
16-16.5	17-18
17-19	19-22

Figure 1

Phase micrograph of an 11.5dpc testis cultured for one day (equivalent to 12.5dpc) in culture medium. A distinct peripheral blood vessel is visible at the edge (arrow). (x150)

Figure 2

Light micrograph of a 13dpc testis fixed immediately on removal. Note developing testicular cords (TC) consisting of irregularly-shaped pre-Sertoli cells (S) enclosing large, round germ cells (G). (X300)

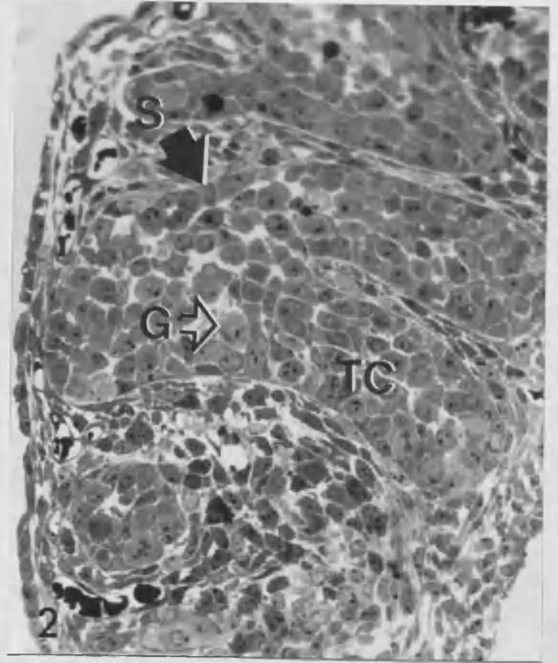
Figure 3

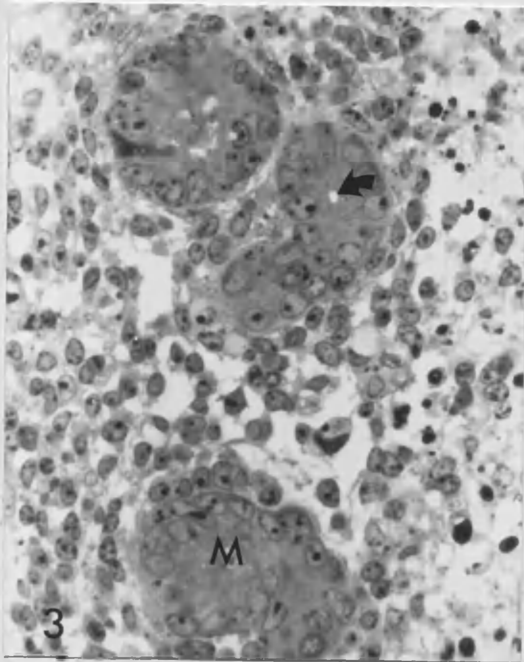
Light micrograph of mesonephric tubules (M) consisting of cuboidal epithelial cells. The lumen is obvious in one mesonephric tubule (arrow). (X450)

Figure 4

Phase micrograph of a 13dpc testis after 2 days culture (equivalent to 15dpc). Note distinctive blood vessels (arrow). Testis is more rounded than that in Fig. 1. (X150)

1


S
G
TC
2


M
3

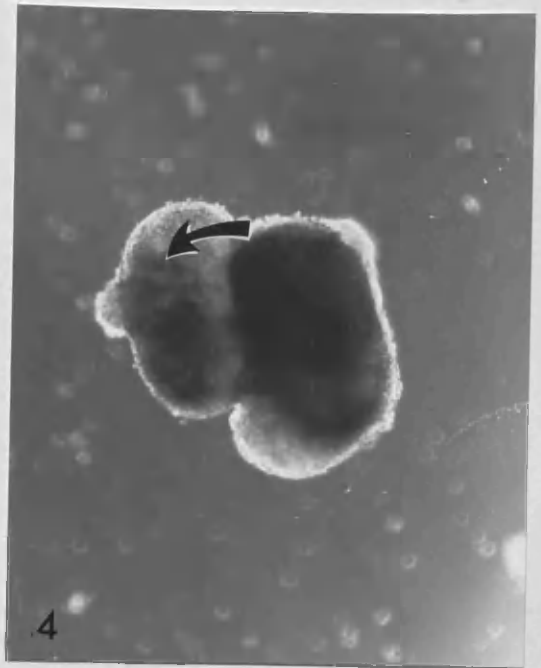

4

Figure 5

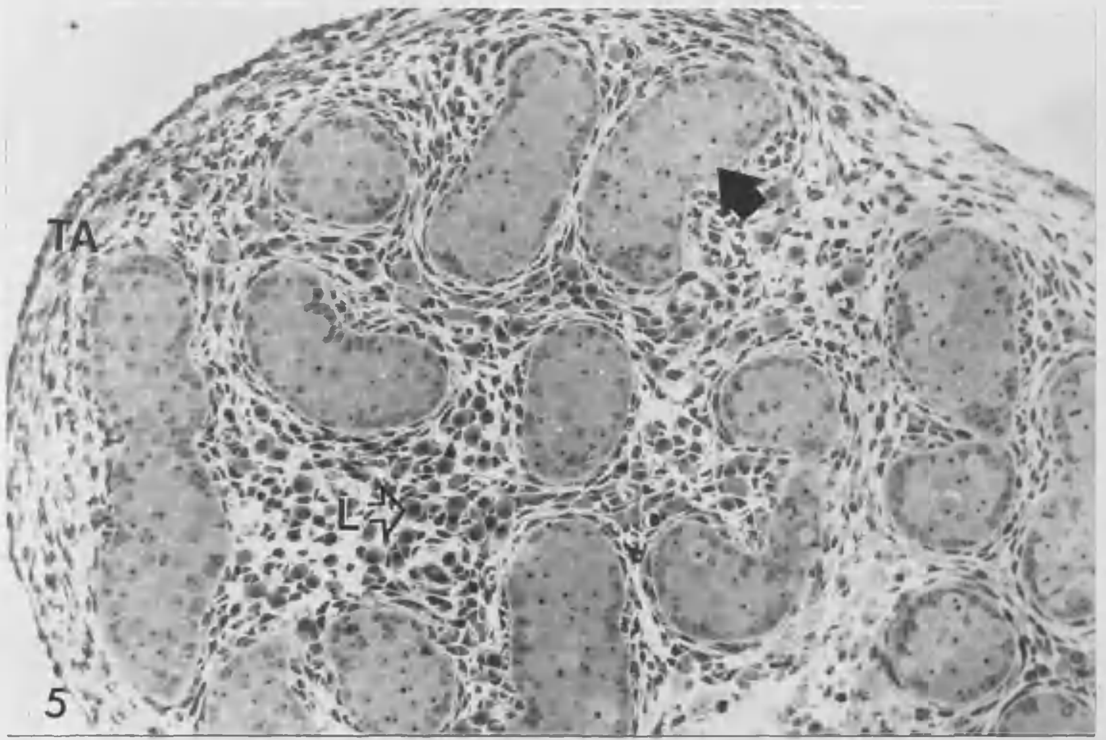
Light micrograph of a testis, fixed immediately on removal from a 17dpc fetus. Testicular cords are well-organised (arrow). Leydig cells (L) have differentiated within the vascular interstitial tissue. A tunica albuginea (TA) is present consisting of several layers of flattened somatic cells. (X200)

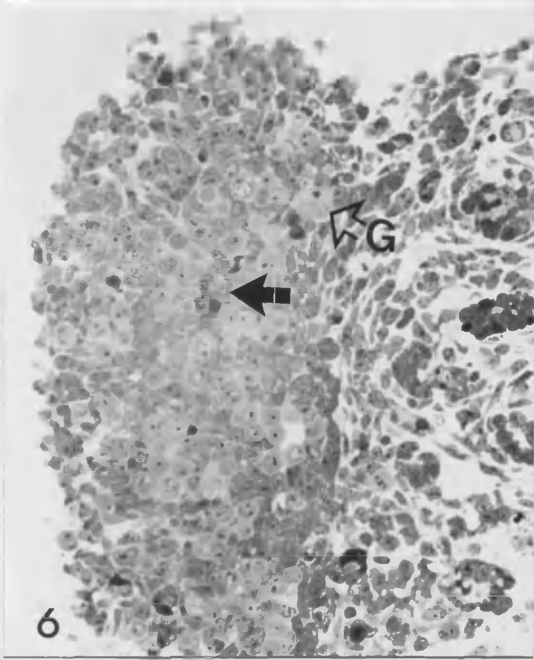
Figure 6

Light micrograph of an ovary, excised and fixed on 13dpc. Note germ cells within indistinct ovigerous cords (G) outlined by flattened somatic cells (arrow). (X250)

Figure 7

Phase micrograph of floating ovary excised on 13dpc and cultured for 2 days. Note the relatively homogeneous appearance of the tissue in comparison to Fig. 4. The Wolffian (W) and Müllerian (M) ducts can be seen at the edge of the mesonephros. (X150)


TA
L
5


G
6

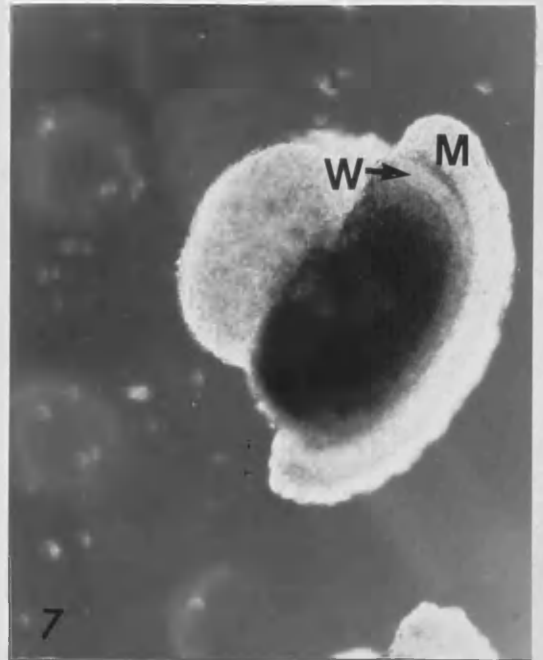
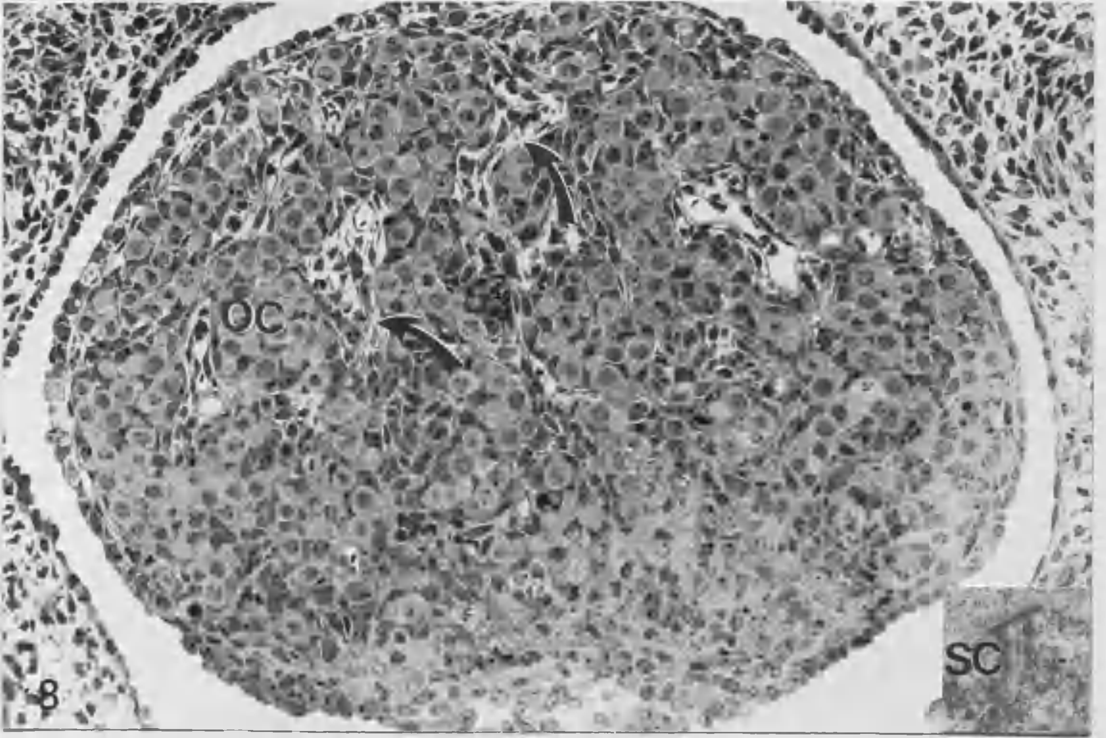

W
M
7

Figure 8

Light micrograph of a 17dpc ovary consisting of oocytes in the pachytene stage of meiotic prophase arranged in distinct ovigerous cords (OC), with vascular interstitial tissue containing connective tissue septa (arrows). (X250). Inset shows a synaptonemal complex (SC). (X 21,000)


OC
SC
8

CHAPTER 3 : RESULTS

The results are divided into 6 sections:-

1. Gonad cultures in testis-conditioned media
2. Gonad cultures in ovary-conditioned media
3. Gonad cultures in adrenal gland-conditioned media
4. Isolated germ cell cultures in ovary- and testis-CM
5. Electrophoresis and protein analysis of CM
6. Observations during the culture period

SECTION ONE - testis-conditioned media

Testis-CM were generated from testes removed between 13dpc and 15dpc and cultured for 2 days (Table 2).

Table 2

Testis-conditioned media

Expt. No.	Age of conditioned medium (dpc)	Age/Sex* cultured explants	Duration of culture (days)	Total No. paired explants cultured	No. Examined Experimental	No. Examined Control
1.1	13-15	13F	4	11	11	10
	13-15	13F**	4	6	2	1
	13-15	13F***	4	9	9	9
	13-15	13F	7	5	1	1
1.3	13-15	12F	2	6	6	6
1.4	14-15	12.5F	4	7	7	7
1.5	15-17	12F	2	6	4	4
1.6	15-18	12M	2	6	6	6
1.7	17-19	11.5	7	3	3	3
1.8	17-19	13	2	3	3	3

* F = Female, M = Male
** Heat-inactivated CM
*** Filtered CM
Necrosis was limited in all explants, although slightly more extensive in cultures maintained for 7 days.

Experiments 1.1, 1.3, 1.4, 1.5 and 1.7 examined with TEM, others with light microscope only.

1.1

A total of 11 ovaries, excised on 13dpc, were cultured in 13-15dpc testis-CM for 4 days and analysed by light and electron microscopy. In addition, the total number of germ cells and gonadal volume was determined in 11 experimental and 10 control ovaries. Additional control cultures utilised the same CM following heat-inactivation and consequent denaturation of any proteins which had diffused into the medium. In presenting this experiment, control and experimental results shall be presented separately due to the marked effects induced by CM.

Control cultures

During the 4 day culture period, control ovaries had increased in size and were more rounded in appearance (Fig. 9). Ovarian morphology resembled that of a slightly earlier stage than 17dpc (approximately 16-17dpc). However, meiosis had progressed to the pachytene stage of prophase I with synaptonemal complexes evident within oocyte nuclei resembling those of the 17dpc *in vivo* ovary (Fig. 13). Mitotic figures were occasionally seen at, or near to, the periphery of the ovary.

Experimental cultures

Initial examination of ovaries cultured in 13-15dpc testis-CM indicated that the number of oocytes was reduced (Fig. 10). This was confirmed following quantitative analysis of serially sectioned ovaries with a

statistically significant (one-tailed Student's t-test) reduction in oocyte number (713 ± 177) as compared to cultured controls (1218 ± 166) (Table 3). Gonadal volume was also estimated in 10 out of 11 ovaries and results showed that 7 of these were smaller than controls, although this was not statistically significant.

An additional control consisted of 6 ovaries, cultured in 13-15dpc testis-CM which had been heat-inactivated, with controls cultured in 13-15dpc testis-CM as above. Two experimental ovaries (in heat-inactivated CM) and one control (in non-activated CM) were serially sectioned and the number of oocytes estimated as above. Those ovaries cultured in heat-inactivated CM contained similar numbers of oocytes (1234) to ovaries cultured in non-conditioned medium. Ovarian morphology did not differ from those cultured in the previous experiment (Fig. 11).

Within ovaries cultured in 13-15dpc testis-CM, oocytes were frequently grouped into small ovigerous cords, which were loosely organised. In those ovaries with very few oocytes, these often lay singly or in pairs. Organisation of ovarian structure had proceeded, even though the full complement of oocytes was absent, with connective tissue septa obvious in the vascular interstitial tissue (Fig. 16). Most of the somatic cells appeared healthy, and made up the bulk of the tissue. As in cultured controls, mitotic figures were occasionally

seen at, or near to, the periphery of the ovaries. Meiosis was not inhibited by the 13-15dpc testis-CM, as evidenced by the presence of distinct synaptonemal complexes in oocyte nuclei (Fig. 14).

Examination of Müllerian ducts revealed no sign of regression and mitotic cells were occasionally seen (Fig. 15). Ducts developed normally in both control and conditioned medium. In addition, the Wolffian ducts showed signs of regression being consistently smaller in diameter and often containing dark amorphous granular material within the lumens.

<u>13dpc ovaries cultured in filtered 13-15dpc testis-CM for 4 days</u>

Testis-CM was filtered prior to culture for 4 days of 9 ovaries. Ovaries were examined morphologically and appeared similar to those cultured in unfiltered CM (Fig. 12).

It is concluded that the inhibiting factor present in 13-15dpc testis-CM is not retained by 0.22μm Millipore filters.

<u>13dpc ovaries cultured in 13-15dpc testis-CM for 7 days</u>

A further experiment was carried out to examine whether ovarian differentiation would proceed in the CM to the beginning of follicle formation.

A total of 5 ovaries were cultured in 13-15dpc testis-CM for a prolonged period of 7 days. At the end of 7 days in CM, both control and experimental ovaries

appeared healthy and had settled and extended fibroblast-like cell sheets peripherally. One experimental and one control ovary was sectioned and examined using the light microscope.

Ovarian differentiation proceeded in both experimental (Fig. 17) and control (Fig. 18) cultures to approximately 18 and 19dpc, respectively.

Oocytes were enlarged in both the experimental and control ovaries examined, and pre-follicle cell processes had separated many within developing unilaminar follicles. Although not examined with the electron microscope, oocyte nuclei appeared to be in the late stages of meiotic prophase.

The healthy appearance of experimental ovaries under the phase microscope was confirmed histologically. There was no necrotic centre, although more necrotic cells were evident than within the control. It should be noted that CM was generated over a 2 day culture period in addition to the 7 days with ovaries. It would be advisable in future studies to replenish CM during prolonged periods of culture. Isolation of individual oocytes by pre-follicle cell processes was more pronounced in the control culture.

In conclusion, ovaries continued development and appeared healthy when examined with the inverted phase contrast microscope _in vitro_. However, 13-15dpc testis-CM had a marked effect on germ cell number, with approximately half those in control cultures after 4 days. Ovaries were

more rounded, even though all had settled during the 7 day culture period. Organisation of somatic tissue was not adversely affected and had continued in both controls and experimentals. Histological examination revealed that follicle development had been initiated in both the experimental and control ovaries examined.

Table 3

Quantitative Analysis of Cultured Ovaries (± SEM)

	NUMBER OF OVARIES	MEAN OOCYTE NUMBER	MEAN GONADAL VOLUME (mm3)
CONTROL	10	1218 ± 166	0.018 ± 0.002
EXPERIMENTAL (Testis-CM)	11	713 ± 177*	0.018 ± 0.001**
HEAT-INACTIVATED (Testis-CM)	2	1234	not measured

* $P < 0.05$ ($t = 2.09$) as compared to controls using a one-tailed Student's t-test.

** Not significantly different as compared to controls.

1.2

Indifferent ovaries and testes cultured in 13-15dpc testis-CM for 7 days

Three ovaries and 3 testes were excised at 11.5dpc and cultured for 7 days in 13-15dpc testis-CM. As ovaries are usually delayed in development in prolonged cultures by approximately 48 hours, oocytes at a stage of meiosis equivalent to those of a 16-17dpc ovary with synaptonemal complexes evident within nuclei might be expected (Mackay and Smith, 1986).

Results proved inconclusive as gonadal development had not advanced in either the control or experimental cultures and cultures are not, therefore represented on Table 2.

In conclusion, 13-15dpc testis-CM produced a statistically significant reduction in oocyte number of 13dpc ovaries cultured 4 days. Three days further culture indicated that the follicular development of remaining oocytes was unaffected. The diffusible substance responsible for the observed reduction was removed from the CM by heat-inactivation but not by filtration with 0.22μm Millipores.

1. 3

12dpc ovaries cultured in 13-15dpc testis-CM for 2 days

Due to the poor development of indifferent ovaries in 13-15dpc CM for 7 days, a total of 6 ovaries were cultured

for a shorter period of 2 days.

Difficulties arose in the analysis of 12dpc gonads which had been cultured for only 2 days. The amnion sexing procedure is 80-90% accurate; however, testes and ovaries cultured for such a short period often appeared morphologically similar. Expanded blood vessels were often noted around clusters of germ cells which, if within somatic tissue, would normally be identified as developing testicular cords with flattened cells outlining peripheries. Further, the *in vivo* testis develops larger blood vessels than the equivalent ovary by 12.5dpc. However, such cords may have formed as a direct consequence of the abnormal expansion of blood vessels and may indeed be a natural initial response to removal of a supportive framework of cellular tissue. The flattened cells may be endothelial cells which would have a similar appearance to those cells characteristically seen around developing testicular cords. Consequently testes could be identified if two layers of nuclei were visible around cord formations.

Ovaries had become more rounded than at the time of excision and appeared healthy. Clusters of germ cells were surrounded by the vascular somatic tissue. Large, expanded blood vessels were occasionally present within both control (Fig. 19) and experimental cultures. As a result, a reduction in germ cell number could not be confirmed, although a slight reduction seemed evident in one experimental ovary in which smaller blood vessels developed

(Fig. 20). In other respects, morphology was similar in both culture conditions, resembling that of the 13-14dpc _in vivo_ ovary. Mitotic figures were noted, both within the surface epithelium and within the underlying tissue. As in other 2 day cultures of gonads excised on 12dpc, the Müllerian duct was not obvious although complete serial sectioning of tissue could have revealed the developing duct. Müllerian ducts may not have continued development _in vitro_. Dyche (1979) has stated that both pairs of ducts are present at 12dpc. However, the staging of fetuses differed slightly from that used in the present study. Regression of the appropriate ducts, in both males and females, commences at around 13dpc _in vivo_ (according to Dyche, 1979). It would, therefore, be expected that there would be little sign of regression within cultured ovaries. In this experiment, there was no sign of incipient tunica albuginea formation beneath the surface epithelium (Table 4).

In conclusion, 2 day culture in 13-15dpc testis-CM had no apparent effect on 12dpc ovaries.

1.4

12.5dpc ovaries in 14-15dpc testis-CM for 4 days

A total of 7 ovaries, excised on 12.5dpc, were cultured in 14-15dpc testis-CM (generated as for isolated germ cells) for 4 days.

Ovaries had continued to develop both in control (Fig.

21) and conditioned medium (Fig. 22). Germ cells increased in number during the 4 day culture period and the majority were organised within developing ovigerous cords by connective tissue septa and small blood vessels, with a few germ cells isolated within developing unilaminar follicles. There appeared to be fewer germ cells in those ovaries cultured in CM as compared to controls, although both control and experimental ovaries were retarded in development by approximately 0.5 to one day when compared to the *in vivo* 16-17dpc ovary. Pre-follicular cell processes were beginning to separate adjacent oocytes, which were in the pachytene stage of meiosis (Inset, Fig.22).

A possible masculinising effect was observed in 2 out of 7 experimental ovaries with the formation of areas resembling the developing tunica albuginea of a 15dpc testis, (consisting of 3-4 layers of flattened mesenchymal cells and collagen underlying a flattened epithelium) (Fig. 23; Table 4). Control ovaries were covered by a cuboidal epithelium. Regression of the Müllerian duct was not noted in experimentals.

In conclusion, ovarian development proceeded in the 14-15dpc testis-CM and meiosis progressed to a stage equivalent to the 16-17dpc ovary.

1.5

Ovaries, excised on 12dpc, were cultured in 15-17dpc testis-CM for 2 days

A total of 6 ovaries were cultured in 15-17dpc testis-CM for 2 days. Ovaries were well-maintained and had increased in size and become more rounded in both experimentals and controls. Organisation of ovaries occurred for cultures in CM and standard medium. Oogonia had continued to divide mitotically and were arranged in irregular and indistinct ovigerous cords, outlined by the connective tissue septa and small blood vessels (Fig. 24). Experimental ovaries contained expanded blood vessels. A cuboidal epithelium was present in the majority of ovaries examined, although one out of 4 experimental ovaries had developed a tunica albuginea-like structure (Fig. 25; Table 4).

The Müllerian duct was not obvious in sections taken, The Wolffian duct showed signs of regression, with dark amorphous material present within the lumen in one experimental ovary. As the Müllerian duct is incompletely formed at 12dpc, it is probable that in vitro conditions were not optimal for continued development.

1.6

Testes, excised on 12dpc were cultured in 15-18dpc testis-CM for 2 days

A total of six, 12dpc testes were cultured in 15-18dpc testis-CM for 2 days. At the end of the culture

period, all control gonads consisted of large and rounded cords containing both germ and somatic cells and outlined by blood vessels with little interstitial tissue. Flattened cells lay peripherally around cords. Mitotic figures were occasionally seen both within cords and within the surface epithelium. Connective tissue septa were evident within tissue indicating that organisation was in process (Fig. 26). Only one out of 6 controls showed signs of incipient tunica formation with regions of flattened cells 1-2 layers deep beneath the surface epithelium.

Experimental gonads were more indifferent in appearance. Germ cells were often grouped in small clusters, or isolated within the stromal tissue. Flattened dark somatic cells were sometimes associated with peripheries of groups. Small blood vessels permeated the somatic tissue and connective tissue septa had also developed in between clustered germ cells. Signs of tunica albuginea-like development were noted in two out of 6 testes examined. In one, 1-2 flattened cell layers underlay the flattened surface epithelium. Another appeared to have a thick outer covering, consisting of 3-4 layers of cells which were mainly cuboidal (Fig. 27). As in controls, there were mitotic figures in the surface epithelium and within the gonadal tissue.

In conclusion, two out of 6 experimental testes and one control had tunica-like formation. A thickened capsule-like tunica noted in one experimental resembled

that of later stages in development, although cells were not flattened. There was no apparent beneficial effect on testicular cord formation in experimentals within the older testis-CM. Indeed, experimental testes were slightly inhibited as compared to controls.

1.7

11.5dpc ovaries cultured in 17-19dpc testis-CM

Three ovaries were excised on 11.5dpc and cultured in 17-19dpc testis-CM for 7 days. At 17dpc in the testis, newly differentiated and functioning Leydig cells are present within the interstitium, with increased testosterone synthesis. In addition to testosterone, AMH production by the pre-Sertoli cells would be expected.

All 3 ovaries developed a tunica albuginea-like structure (Fig. 28; Fig. 29; Table 4). Germ cell development continued to the equivalent of a 17dpc ovary, with synaptonemal complexes present within oocyte nuclei similar to those observed in equivalent ovaries. The Müllerian duct, which has not yet formed at 11.5dpc, did not develop *in vitro*.

1.8

13dpc ovaries cultured for 2 days in 17-19 testis-CM

Three 13dpc ovaries were cultured in 17-19dpc testis-CM for 2 days in order to further investigate effects on duct development at the critical period for male duct regression and stabilisation.

Ovarian development proceeded in both control (Fig. 30) and experimental (Fig. 31) cultures to the equivalent *in vivo* stage (14-15dpc). There was no sign of tunica albuginea development (Table 4) and no apparent reduction in germ cell number in those ovaries cultured in CM. Expanded blood vessels were present in both culture conditions. However, in the control these were located at the gonad-mesonephric junctional region, whilst in the experimentals blood vessels had surrounded groups of large pale germ cells and darker small somatic cells. Flattened somatic cells surrounded these groups. Connective tissue septa were evident throughout the tissue in all ovaries examined. Ovaries were more rounded in appearance than at the time of excision, although one ovary had flattened to such an extent during culture that the gonadal region was not obvious. Oogonia were clustered in developing ovigerous cords and connective tissue septa were evident. Germ cells had increased greatly in number and mitotic figures were occasionally present within cords and within the cuboidal surface epithelium, indicating the vitality of the tissue. In both control and experimental cultures, the Müllerian duct was larger than the adjacent Wolffian and contained mitotic figures (Fig. 32).

In conclusion, 17-19dpc testis-CM had an apparent masculinising effect on ovaries removed at the indifferent stage and cultured for 7 days. Germ cells continued along the female pathway, entering meiosis *in vitro* and continuing to the pachytene stage of meiosis.

Ovaries removed one and a half days later (13dpc) and cultured for only 2 days were equivalent to the 15dpc ovary and were not retarded in development. There were no signs of tunica formation in these ovaries. Distinctive expanded blood vessels, commonly seen in 12dpc gonads cultured for 2 days, had surrounded groups of germ cells and somatic cells, with more flattened somatic cells around these, which are probably endothelial. These 'cords' resembled the disorganised testicular cords seen in other cultures. Ducts developed normally with no apparent Müllerian inhibiting or Wolffian maintaining effect.

Table 4

Tunica albuginea development in experimentally cultured ovaries

	PERIOD OF TESTIS-CM GENERATION (dpc)						
	13-15			14-15	15-17	17-19	
DURATION OF CULTURE (DAYS)	2	4	7	4	2	2	7
AGE OF OVARIES							
11.5	-	-	-	-	-	-	3(3)
12	0(6)	-	-	-	1(4)	-	-
12.5	-	-	-	2(7)	-	-	-
13	-	0(11)	0(1)	-	-	0(3)	-

(n) = number of explants examined

\- = experiment not carried out

Tunica albuginea development was not noted in any of the control ovaries (for numbers see Table 2).

Table 4

Tunica albuginea development in experimentally cultured ovaries

	PERIOD OF TESTIS-CM GENERATION (dpc)						
	13-15			14-15	15-17	17-19	
DURATION OF CULTURE (DAYS)	2	4	7	4	2	2	7
AGE OF OVARIES							
11.5	-	-	-	-	-	-	3(3)
12	0(6)	-	-	-	1(4)	-	-
12.5	-	-	-	2(7)	-	-	-
13	-	0(11)	0(1)	-	-	0(3)	-

(n) = number of explants examined

- = experiment not carried out

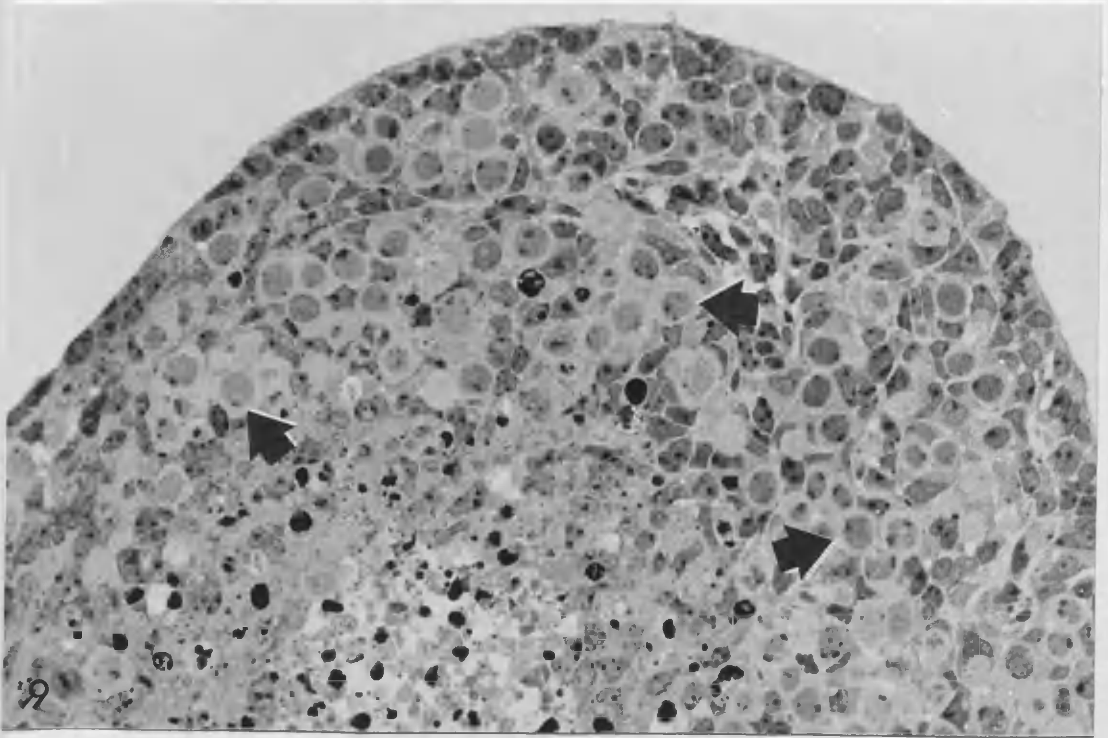
Tunica albuginea development was not noted in any of the control ovaries (for numbers see Table 2).

Figure 9

Control ovary removed on 13dpc and cultured 4 days; oocytes (arrows) are organised in ovigerous cords which resemble those of a 16-17dpc ovary. (X400)

Figure 10

Experimental 13dpc ovary cultured 4 days in 13-15dpc testis-CM. Few oocytes (arrows) present within small ovigerous cords. (X500)


9

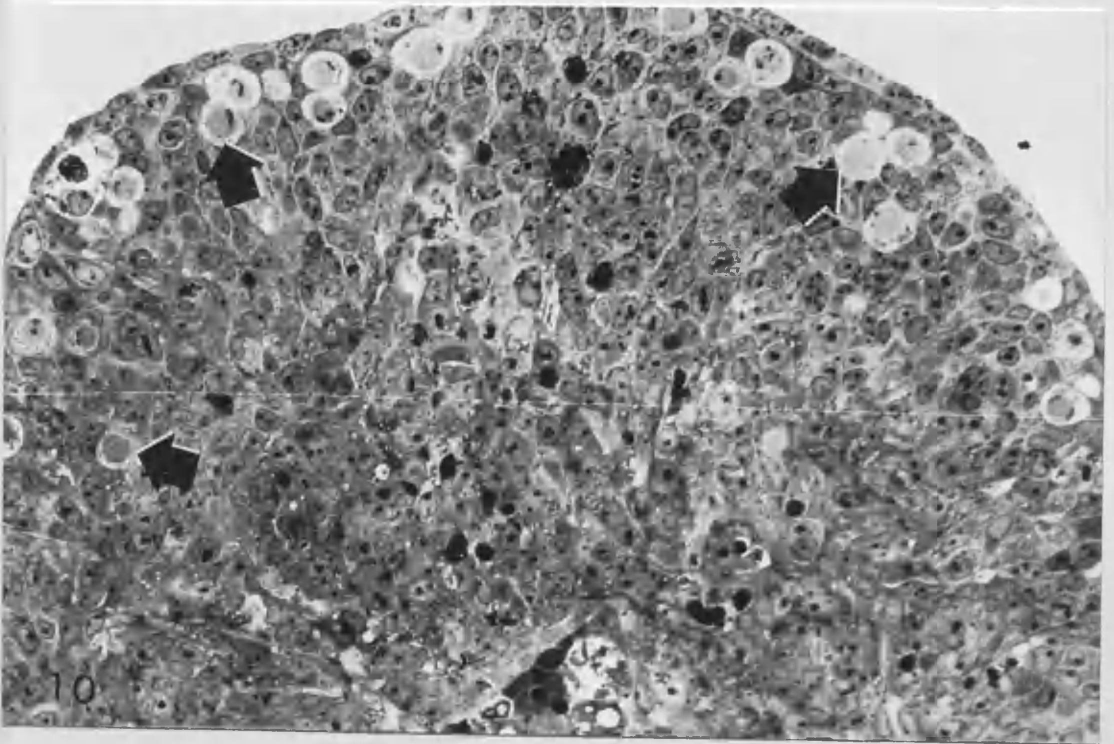
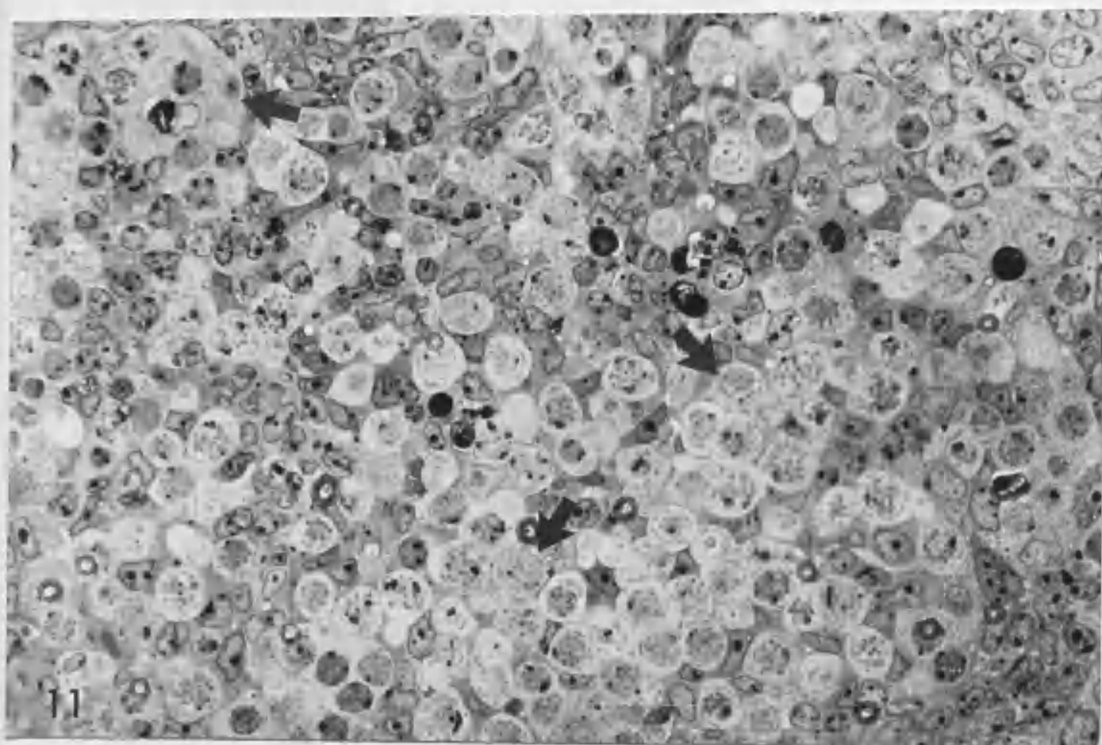

10

Figure 11

Ovary, removed on 13dpc and cultured 4 days in heat-inactivated 13-15dpc testis-CM, resembling the control ovary in Fig. 9. Numerous oocytes are enclosed within developing ovigerous cords (arrows). (X550)

Figure 12

13dpc ovary cultured 4 days in filtered 13-15dpc testis-CM which appears similar to ovary cultured in unfiltered testis-CM. (X500)


11

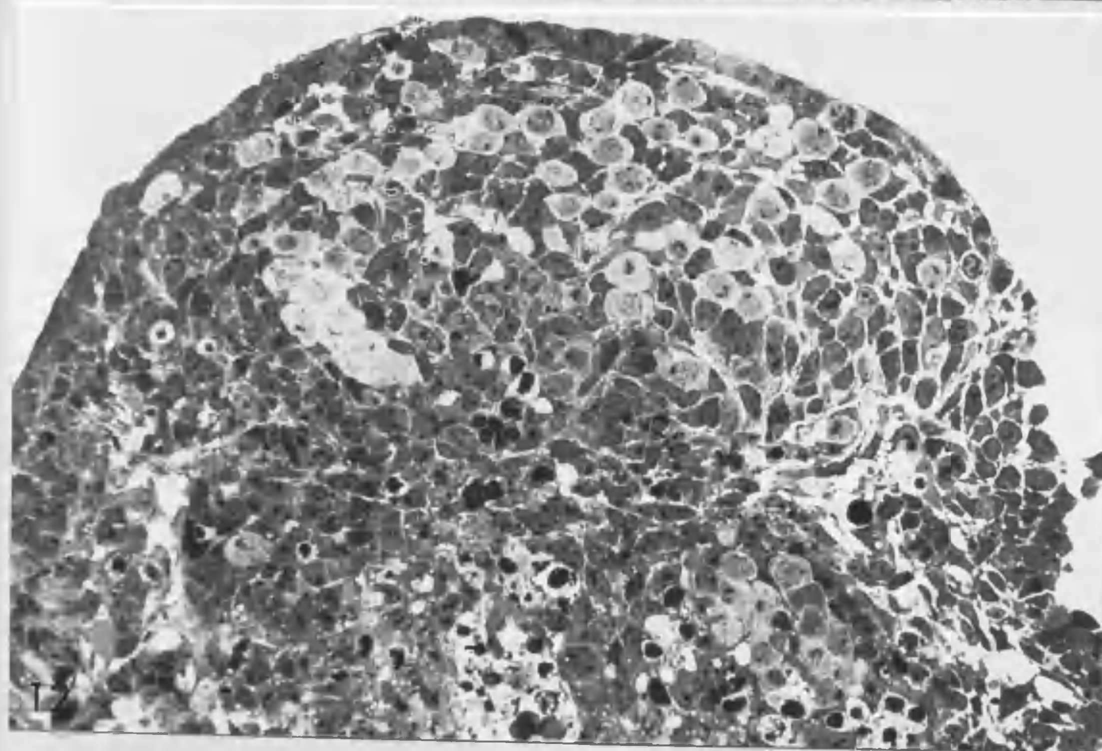
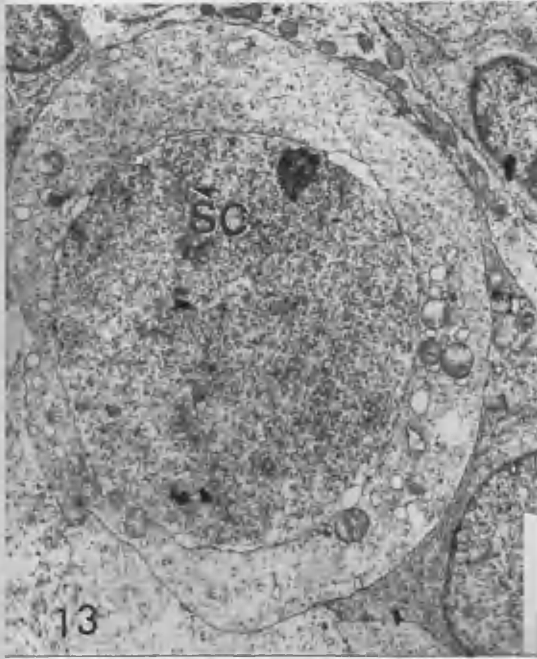

12

Figure 13

Electron micrograph of control 13dpc ovary cultured 4 days; note prominent synaptonemal complex (SC) in oocyte nucleus. (X5000)

Figure 14

Electron micrograph of experimental 13dpc ovary cultured 4 days in 13-15dpc testis-CM; oocyte nucleus contains synaptonemal complexes (arrows). (X5000)


SC
13

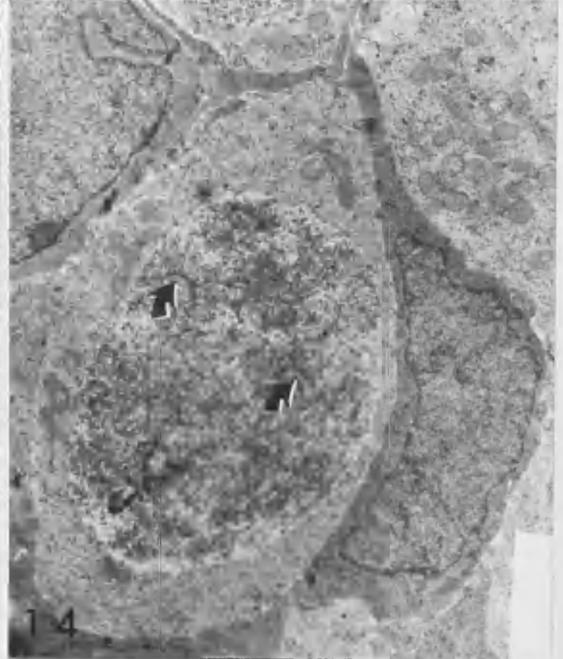
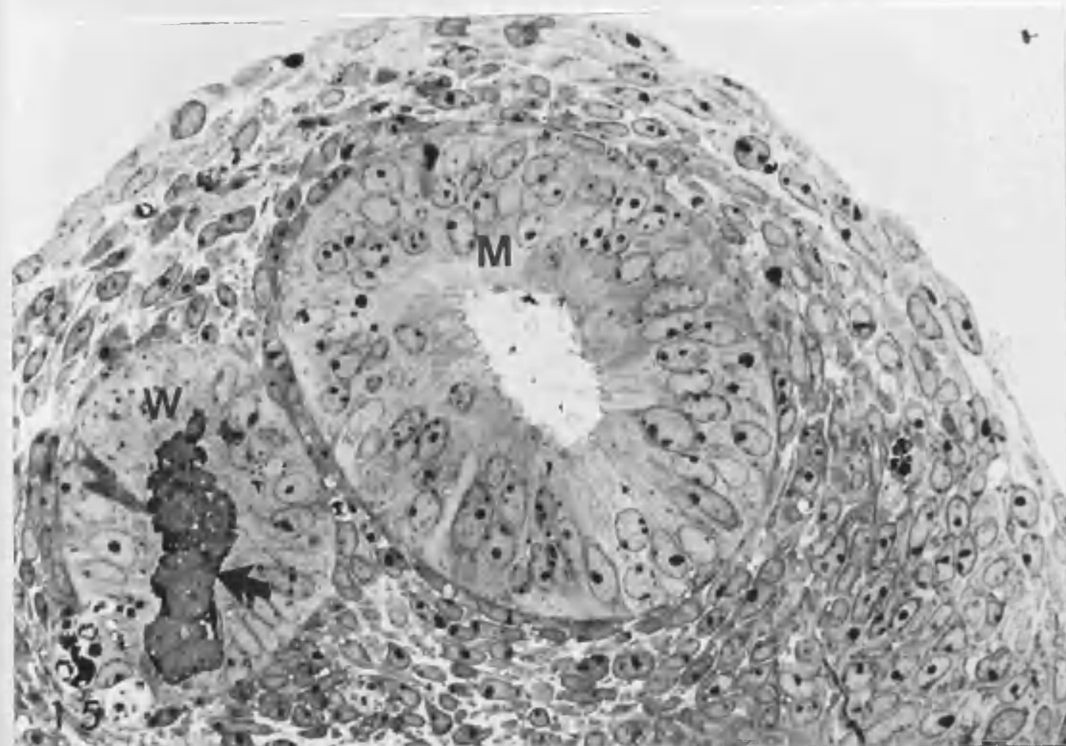

14

Figure 15

Müllerian (M) and Wolffian (W) ducts in experimental ovary, removed on 13dpc and cultured 4 days in 13-15dpc testis-CM. Wolffian duct lumen occluded with dense amorphous debris (arrow). (X650)

Figure 16

Electron micrograph showing development of connective tissue septa within experimental ovary removed on 13dpc and cultured 4 days in 13-15dpc testis, with flattened fibroblasts (F) and collagen fibres (arrow). (X8400)


M
W
15

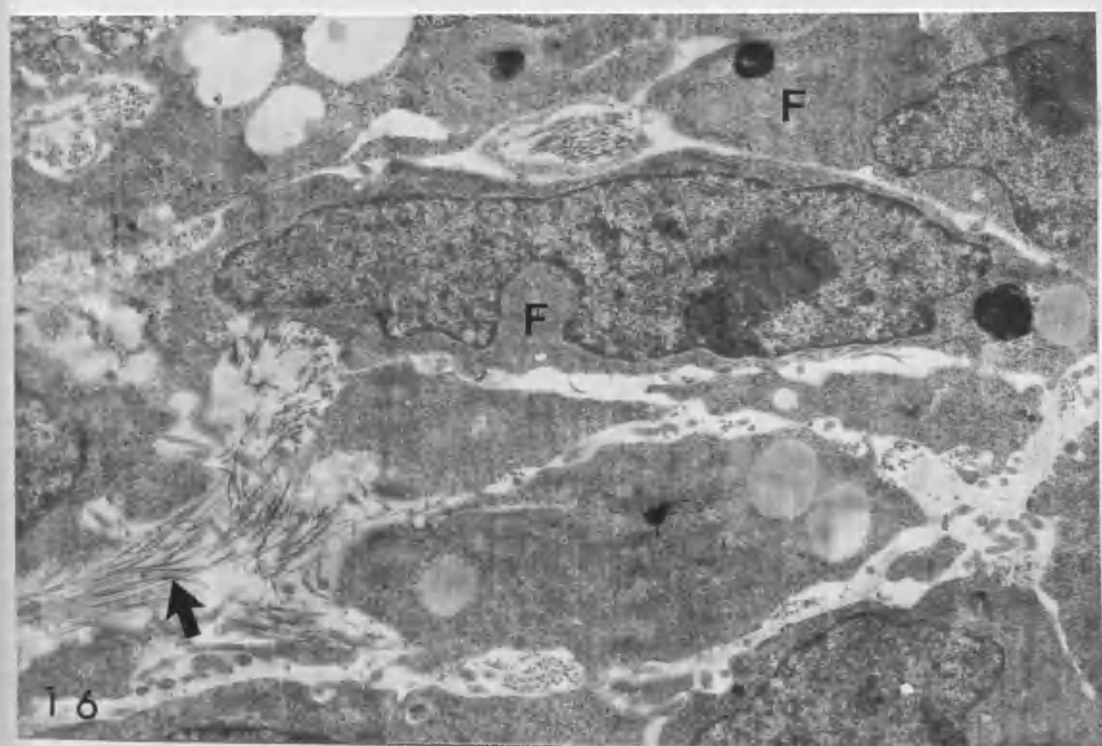

F
F
16

Figure 17

Experimental ovary removed on 13dpc and cultured for 7 days in 13-15dpc testis-CM. Enlarged oocytes are becoming enclosed within developing follicles (arrows). (X600)

Figure 18

Control ovary, removed on 13dpc and cultured 7 days; follicular cell processes have enveloped enlarged oocytes (arrows). (X600)

Figure 19

Control ovary removed on 12dpc and cultured 2 days. Expanded blood vessels surround clusters of germ cells (arrows). (X150)

Figure 20

Experimental ovary removed on 12dpc and cultured 2 days in 13-15dpc testis-CM. Small clusters of germ cells are becoming organised within indistinct ovigerous cords (arrows). (X300)

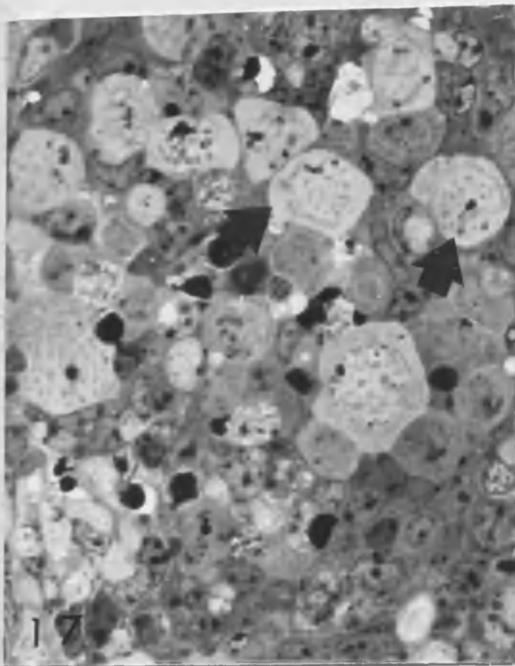
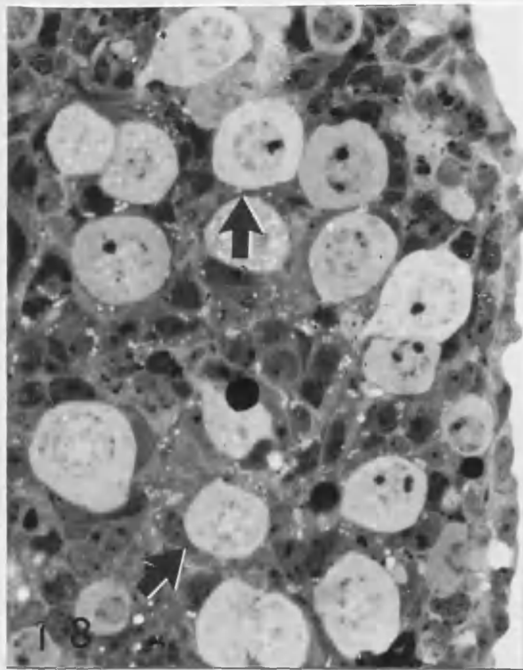
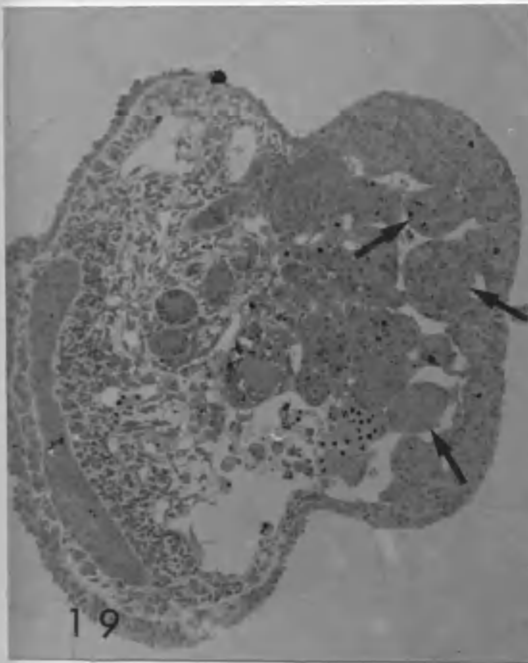
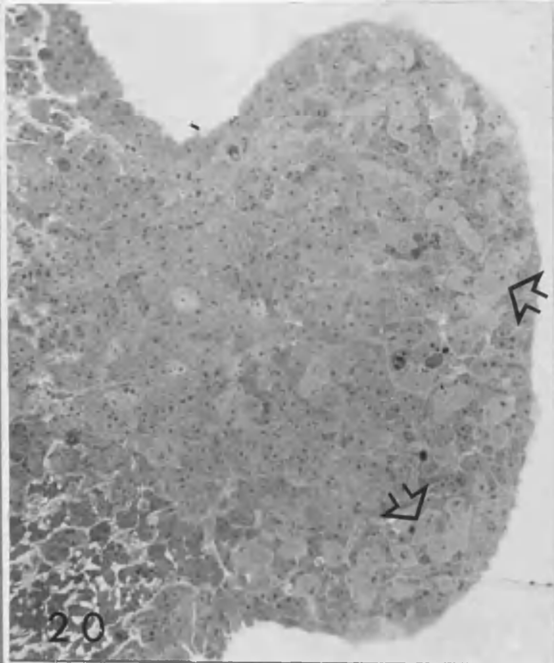
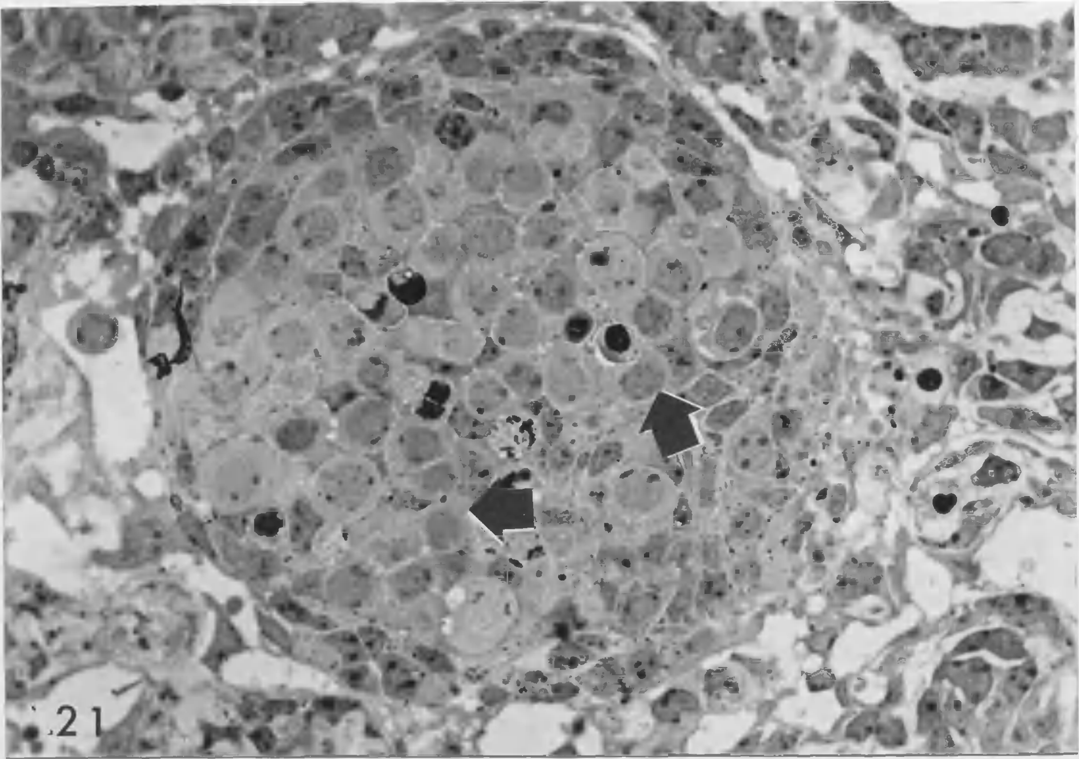

17

18

19

20

Figure 21

Ovary removed on 12.5dpc and cultured 4 days; oocytes are clustered within developing ovigerous cords (arrows). (X800)

Figure 22

Ovary, removed on 12.5dpc and cultured 4 days in 14-15dpc testis-CM. Oocytes (arrows) appear reduced in number and enclosed in irregular ovigerous cords when compared to equivalent control. (X800). Inset shows electron micrograph of a synaptonemal complex indicative of the pachytene stage of meiotic prophase. (X1800)


21

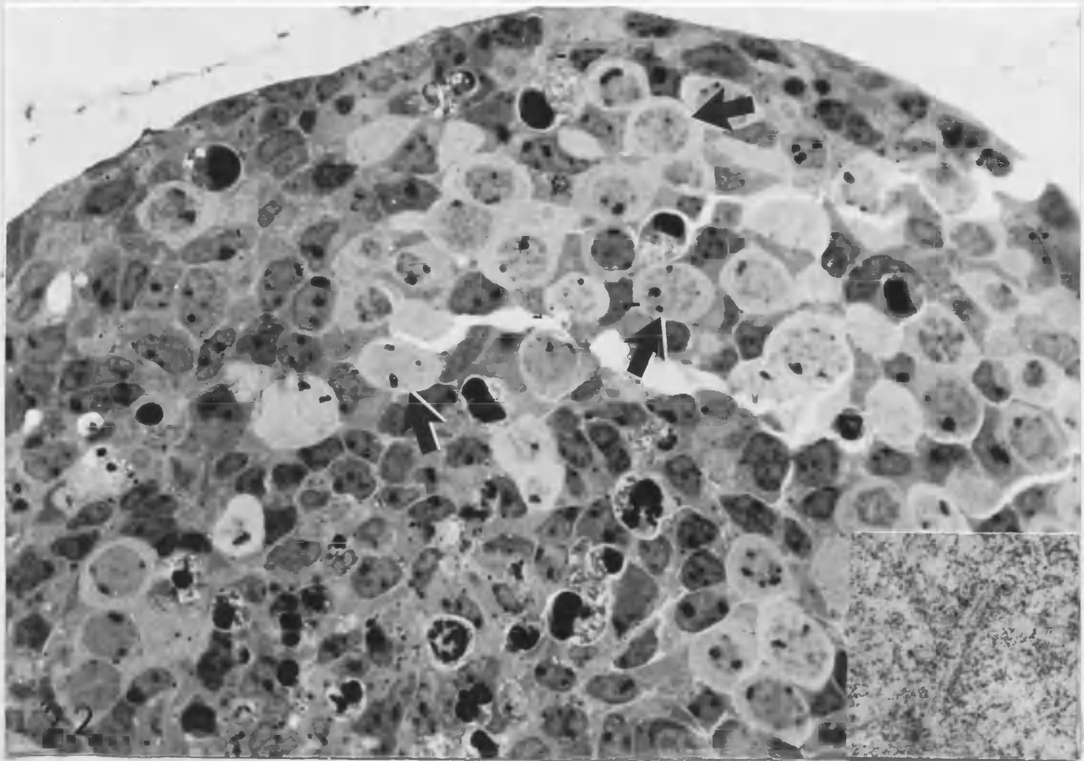


Figure 23

Experimental ovary removed on 12.5dpc and cultured 4 days in 14-15dpc testis-CM. Flattened somatic cells (arrows) resemble the developing tunica albuginea of the 15dpc testis. (X1500)

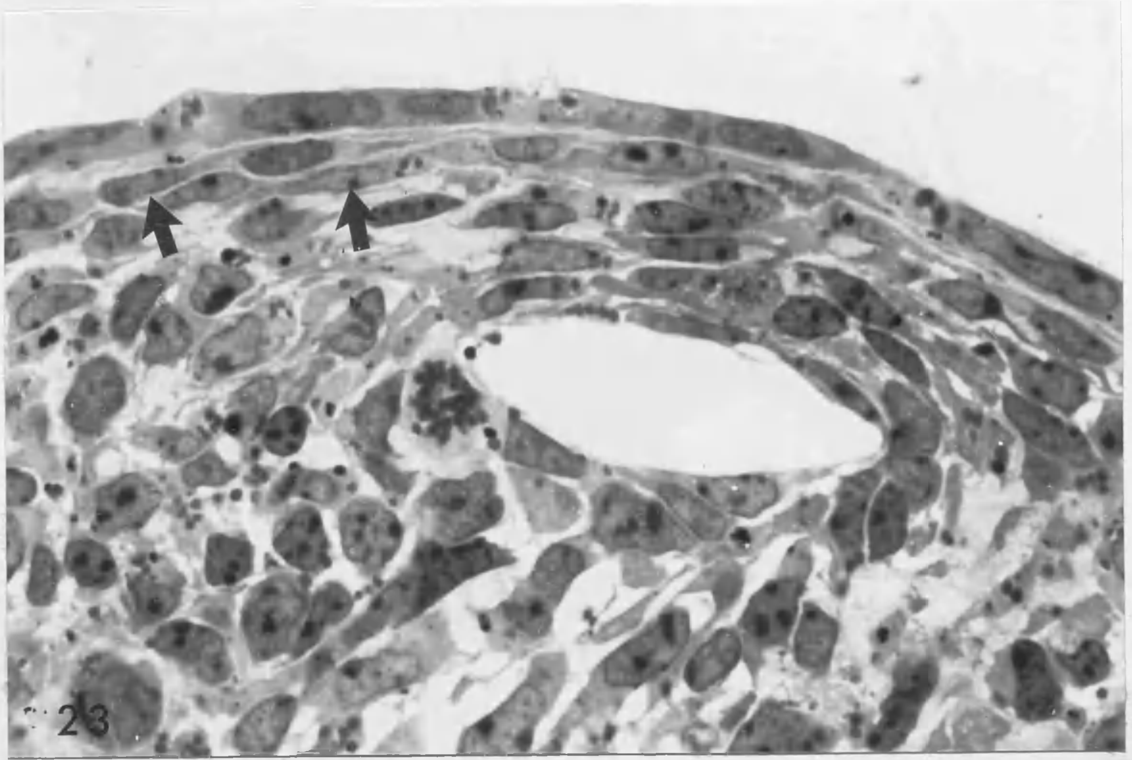

23

Figure 24

Control ovary removed on 12dpc and cultured 2 days. Germ cells are within indistinct ovigerous cords (arrows). Mitotic figues (M) are evident within both the surface epithelium and the ovarian tissue. (X500)

Figure 25

Experimental ovary removed on 12dpc and cultured in 15-17dpc testis-CM for 2 days. Note flattened somatic cells resembling a differentiating tunica albuginea (TA). (X500)

Figure 26

Control testis removed on 12dpc and cultured 2 days. Developing testicular cords are surrounded by 2 layers flattened somatic cells (arrows) and expanded blood vessels. A mitotic cell is present within one cord (M). (X300)

Figure 27

Experimental testis, removed on 12dpc and cultured in 15-18dpc testis-CM for 2 days. Testis appears indifferent with a thickened outer layer of cuboidal somatic cells (arrows). (X200)

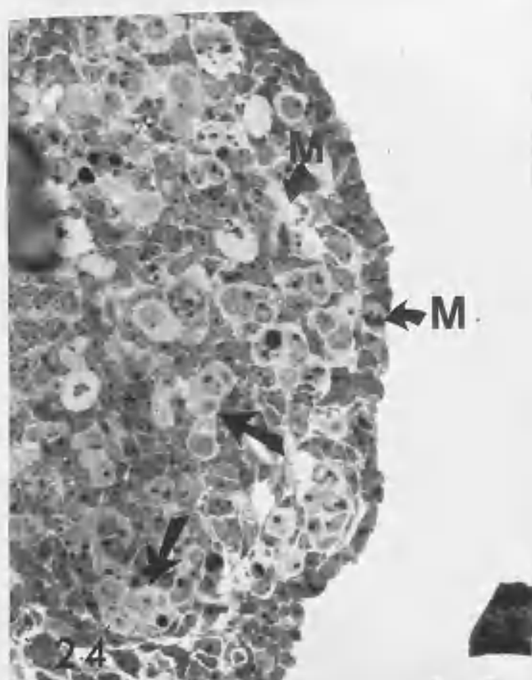
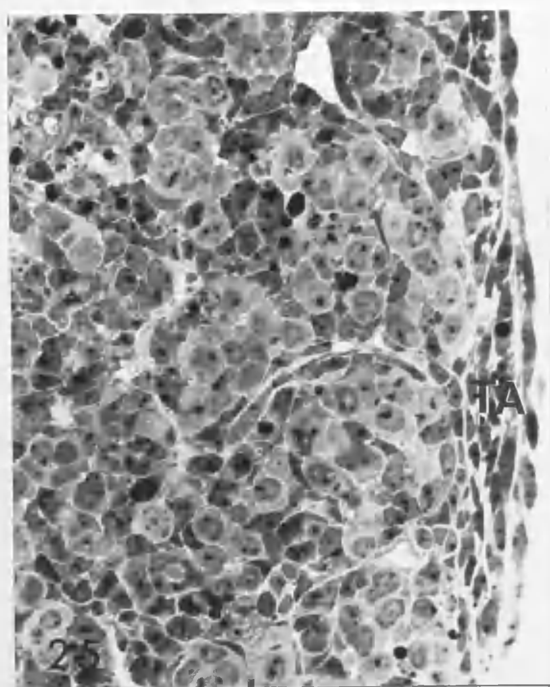
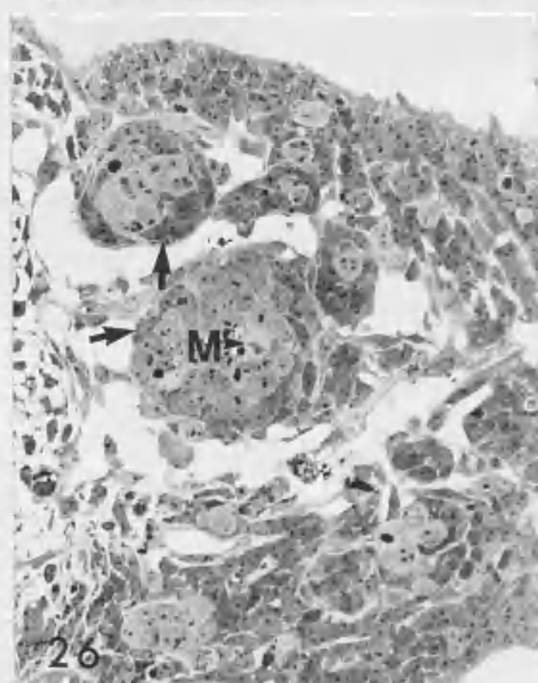
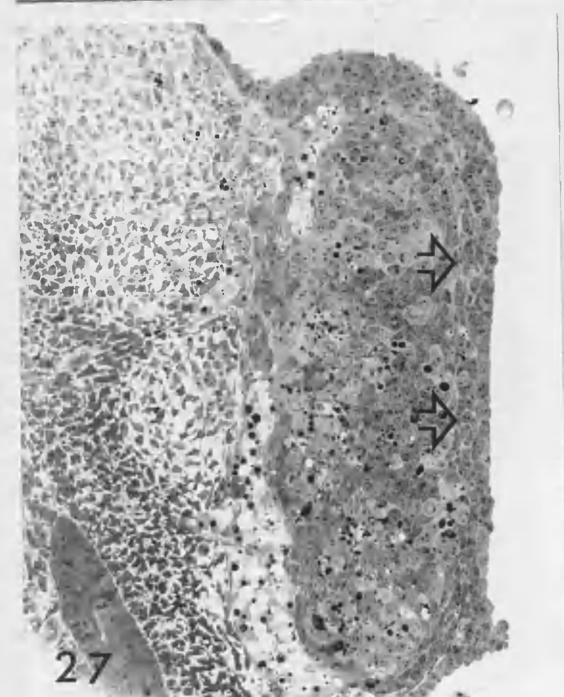
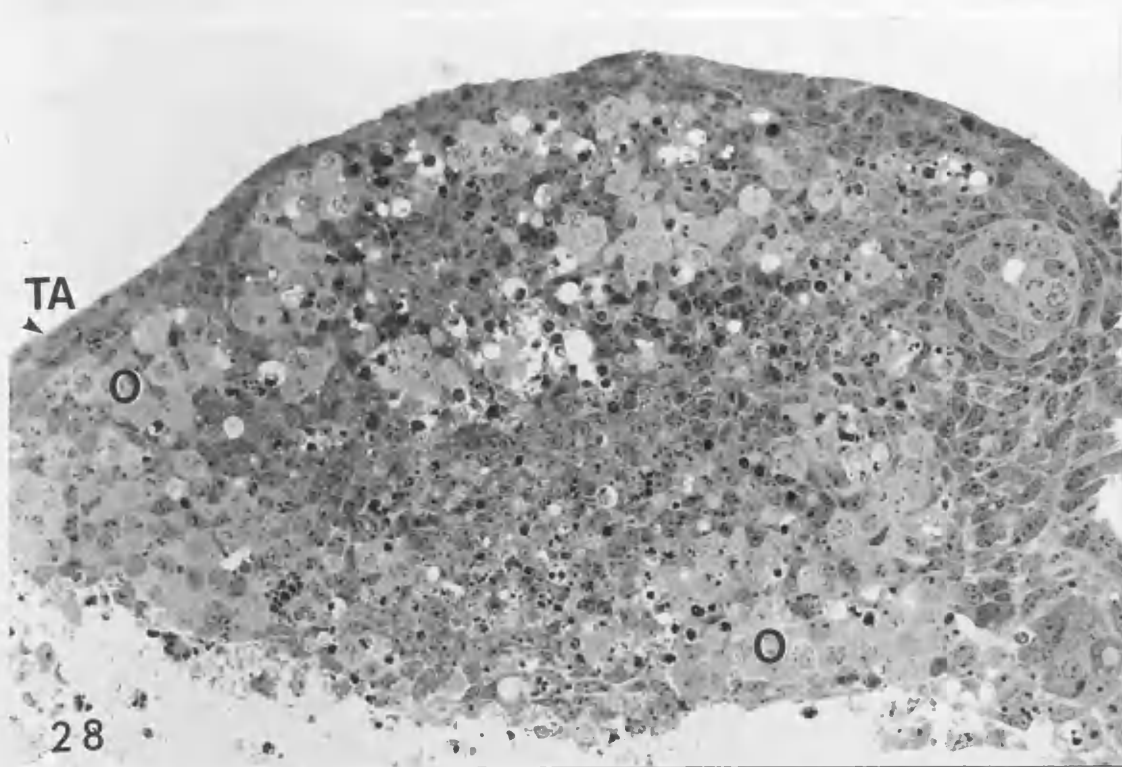

M
M
24

TA
25

Ms
26

27

Figure 28

Experimental ovary, removed on 11.5dpc and cultured 7 days in 17-19dpc testis-CM. Note ovigerous cords (O) and evidence of tunica albuginea development (TA). (X300)

Figure 29

Electron micrograph of experimental ovary removed on 11.5dpc and cultured 7 days in 17-19dpc testis-CM, showing flattened cells at edge resembling the tunica albuginea of a developing testis. (X4200)


TA
O
O
28

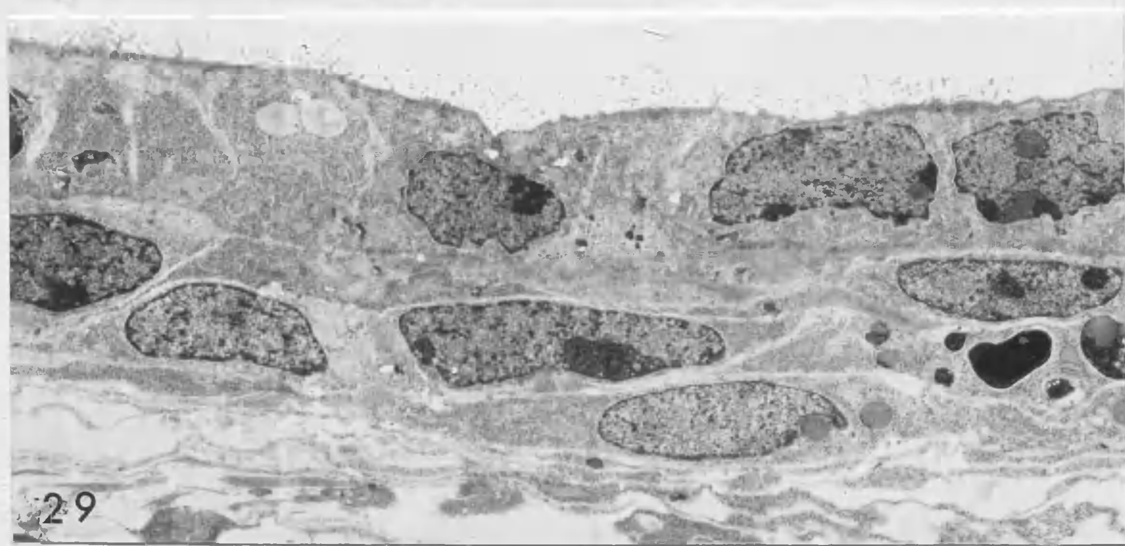

29

Figure 30

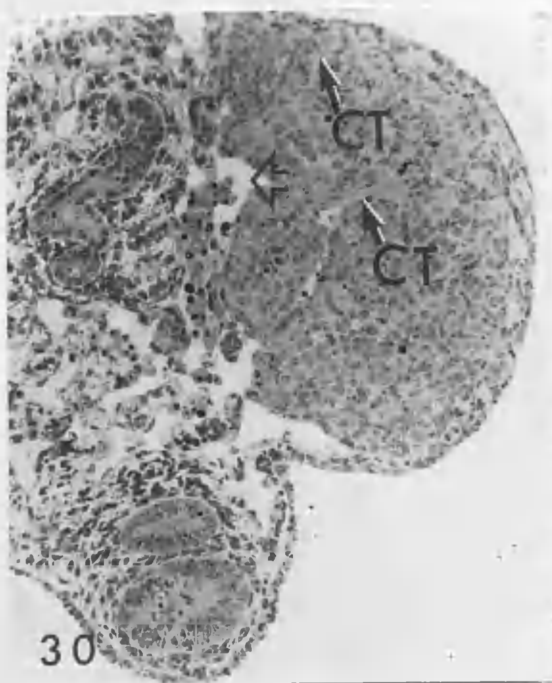
Control 12dpc ovary cultured 2 days. Germ cells are clustered within developing ovigerous cords outlined by connective tissue septa (CT). Expanded blood vessels are obvious at the gonad-mesonephric junction (arrow). (X200)

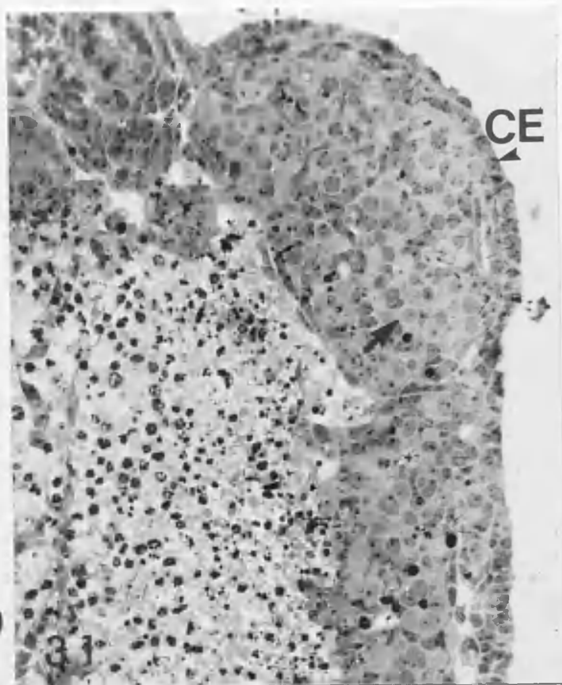
Figure 31

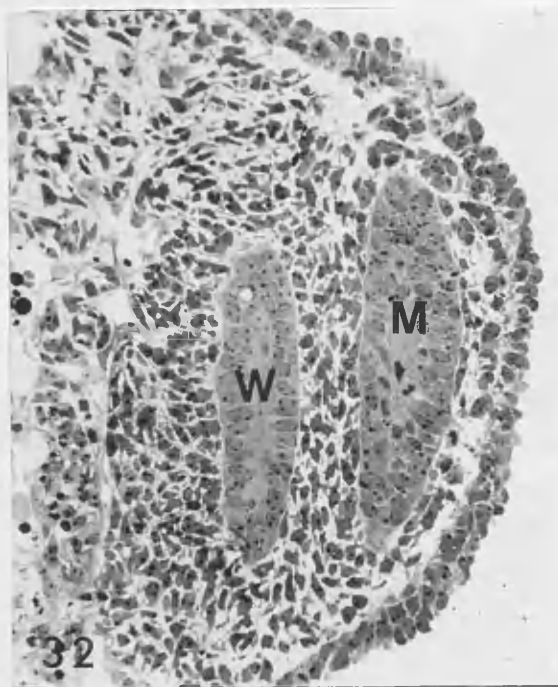
Experimental 12dpc ovary cultured 2 days in 17-19dpc testis-CM. Note cuboidal epithelium covering ovary (C.E.) containing clusters of germ cells within indistinct ovigerous cords (arrow). (X300)

Figure 32

Müllerian (M) and Wolffian (W) ducts associated with experimental 12dpc ovary cultured 2 days in 17-19dpc testis-CM. Mitotic figures are evident within the Müllerian duct. (X300)


CT
CT
30


CE
31


M
W
32

SECTION 2 - Ovary-conditioned media

Conditioned media were generated from ovaries excised between 13 and 15dpc and cultured for 2 days (Table 5).

Table 5

Ovary-conditioned media

Expt. No.	Age of conditioned medium (dpc)	Age/Sex* cultured explants	Duration of culture (days)	Total No. paired explants cultured	No. examined	
					Experimental	Control
2.1	13-15	13M	4	3	2	2
2.2	15-17	13.5M	4	6	6	6
2.3	15-17	14M	4	6	6	6
2.4	15-18	12M	2	3	3	3
		12F	2	1	1	1

F = Female
M = Male

Necrosis was more extensive in 4 day cultures than those maintained for only 2 days, except for 2.3 where necrosis was limited.

Experiments 2.1, 2.2 and 2.3 were examined with the transmission electron microscope (TEM), 2.4 was examined with the light microscope only.

2.1

In Section 1, 1.1, 13-15dpc testis-CM was used to culture 13dpc ovaries for 4 days with significant results. In this experiment 13-15dpc ovary-CM was used to culture 3 13dpc testes for an equivalent period, in order to investigate the effects of putative diffusible substances on entry into meiosis and testicular development.

There were no apparent differences between those testes cultured in 13-15dpc ovary-CM and in control medium (Fig. 33; Fig. 34). Testes had become more rounded during culture. Testicular cord development was disrupted with complete cord breakdown. Deep within the gonads, groups of germ cells indicated the positions of testicular cords present and developing at the start of culture. An interrupted tunica albuginea had developed in 2 out of 3 experimental testes examined. Mitotic figures were occasionally seen within the surface epithelium and within the somatic cells in the outer regions of the testis. The Wolffian duct was well developed. The Müllerian duct was not obvious in sections examined.

In summary, the 3 testes removed at 13dpc and cultured for 4 days did not continue development. Testicular cord integrity was lost in both control and 13-15dpc ovary-CM, suggesting this was a result of prolonged culture. An interrupted tunica albuginea formed in 2 out of 3 experimental testes.

2.2

13.5dpc testes cultured in 15-17dpc ovary-CM for 4 days

A total of 6 testes, excised on 13.5dpc, were cultured in 15-17dpc ovary-CM for 4 days. CM was filtered prior to use. Testicular cord development and structure was not maintained throughout the period of culture (Fig. 35). Aggregates of germ cells indicated where cords had formed at 13.5dpc in both experimentals and controls (Fig. 36). Single germ cells, released from disrupted cords, showed no sign of early entry into meiosis. In one testis examined on the electron microscope, thin cellular processes appeared to have encircled an isolated germ cell (Fig. 37).

Interstitial tissue and centrally located cords had degenerated in the deepest aspects of the urogenital complexes in both cultured control testes and those cultured in CM, although necrosis was less extensive in experimentals. This suggested that the culture conditions were not optimal for this developmental age. In addition, testicular volume is increased by 13.5dpc and a further increase in size was noted during the period of culture which may have resulted in poor diffusion of nutrients into the deepest aspects of the tissue. In some cases, flattened peritubular cells outlined the edges of cords most distal from necrosis. Peripheral interstitial tissue contained small blood vessels, strands of connective tissue and mesenchymal cells. The occasional mitotic figure was also present in experimental testes. Leydig cell

differentiation was not noted in these cultures.

Tunica albuginea development had not advanced in cultured testes, although flattened cells were present underlying some areas of the surface epithelium. A thickened, collagenous capsule had developed around the mesonephros of one urogenital complex cultured in CM.

2.3

14dpc testes cultured in 15-17dpc ovary-CM for 4 days

Six testes, removed half a day later, and cultured in unfiltered 15-17dpc ovary-CM did markedly better during the period of culture, in both controls (Fig. 38) and experimentals (Fig. 39), and resembled a 17dpc testis. All testes had become larger and more rounded in appearance than at 14dpc. The proportion of the total surface area occupied by testicular cords was significantly reduced in testes cultured in CM, as compared to cultured controls, with an accompanying increase in the amount of interstitial tissue (Table 6, p94). Small pre-Sertoli cells, with dark irregularly shaped nuclei lay peripherally, and enclosed the large round pale germ cells within. A basal lamina, lying between flattened peritubular cells and pre-Sertoli cells, is shown at the edge of one testicular cord (Fig. 40).

Differentiated Leydig cells were present in between developing cords. A distinct tunica albuginea was present in all cultured testes, in both CM and control medium, although not as well developed as *in vivo* (Fig. 41).

The Wolffian ducts were well maintained in experimental testes, but in some cases had become greatly expanded so that the cells of the duct wall were attenuated. The adjacent Müllerian duct showed signs of involution (Fig. 42).

In conclusion, 15-17dpc ovary-conditioned media affected the structure of 14dpc testes with a significant reduction in testicular cord surface area, accompanied by an increase in the amount of interstitial tissue. Ovary-CM did not induce meiosis in 13.5 and 14dpc testes cultured for 4 days. 14dpc testes survived and continued development whereas those removed half a day earlier were poorly maintained.

2.4

<u>12dpc testes and ovary cultured in 15-18dpc ovary-CM for 2 days</u>

Two testes were cultured in 15-18dpc ovary-CM with one ovary. Another testis was cultured alone (in both experimental and control conditions).

Testicular cords developed in the two experimental testes cultured with one ovary, but not in the testis cultured alone in 15-18dpc ovarian-CM. All 3 controls contained recognisable cords, which, as in experimentals, were atypical with pre-Sertoli and germ cells lying centrally as well as peripherally <u>in vitro</u> (Fig. 43), reflecting the effects of culture rather than of CM. Flattened somatic cells outlined cords in places. Cords

were closely apposed with little vascular interstitial tissue separating them.

The testis cultured alone resembled an indifferent gonad, with germ cells scattered throughout the tissue (Fig. 44). The contralateral testis, cultured as a control, contained testicular cords. Most germ cells were isolated within the stroma, although small clusters were evident at the junctional region with the mesonephros. Several mitotic figures were present and the tissue was generally healthier than that of the testes cultured together. A distinct cuboidal epithelium covered the gonad, with no signs of tunica-like formation.

Signs of incipient tunica albuginea formation were evident in one out of 3 experimental and control testes, with flattened cells underlying the surface epithelium. Mitotic figures were present in all testes. Both pairs of genital ducts were present, with no signs of involution. The Müllerian duct was small and incompletely developed, however, and probably did not proceed further from the time of excision. The Wolffian duct was larger and fully developed with mitotic figures within the duct wall. At this stage (14dpc) Dyche (1979) reported that a characteristic epithelioid cuff had developed around the male Müllerian duct with an accompanied reduction in diameter.

The ovary cultured with two equivalent testes had continued to develop within the 15-18dpc ovary-CM and was

well-maintained (Fig. 45). The control ovary was similar in appearance. Germ cells had increased in number in vitro within indistinct ovigerous cords. Flattened somatic cells were evident in one area, near the surface epithelium, outlining one germ cell cluster. Small capillaries were present which were smaller than those seen in equivalent testes. Mitotic figures were present both in the surface epithelium and within the ovarian tissue.

Both ducts were present and resembled those within equivalent testes. Again at this stage, Dyche (1979) found that the diameter of the female Wolffian duct was reduced.

The testis cultured alone remained indifferent in structure and did not develop testicular cords which were present in the control. In conclusion, testicular cord development continued in testes cultured together with an ovary in 15-18dpc ovary-CM, although the normal arrangement of pre-Sertoli and germ cells was disturbed, as in controls.

Table 6

Proportional surface area occupied by testicular cords within 6 control 14dpc testes cultured in standard medium and 6 experimental 14dpc testes cultured in 15-17dpc ovary-CM.

PERCENTAGE AREA OCCUPIED BY TESTICULAR CORDS

TESTIS	CONTROLS	EXPERIMENTALS
1	33	19
2	25	20
3	24	19
4	28	25
5	30	22
6	28	25
MEAN % (±SEM)	**28 ± 1.47**	**22 ± 1.25***

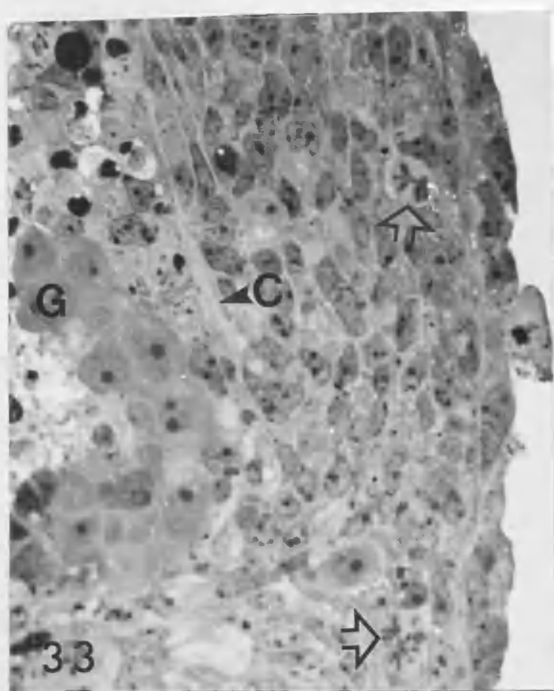
* t=3.59, df=10, $P < 0.005$ using Student's t-test (2-tailed).

Figure 33

Experimental 13dpc testis cultured 4 days in 13-15dpc ovary-CM. Group of germ cells (G) indicates position of original testicular cord. Connective tissue septa are evident within the interstitial tissue (C). Mitotic figures are evident throughout the tissue (arrows). (X700)

Figure 34

Control 13dpc testis cultured 4 days. Clusters of germ cells lie in regions of disrupted testicular cords (arrows). A tunica albuginea is evident (TA). (X700)


G
C
33

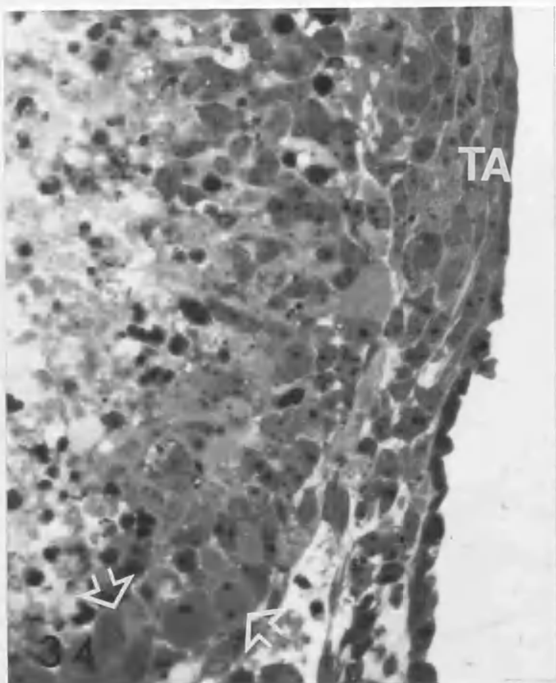

TA
34

Figure 35

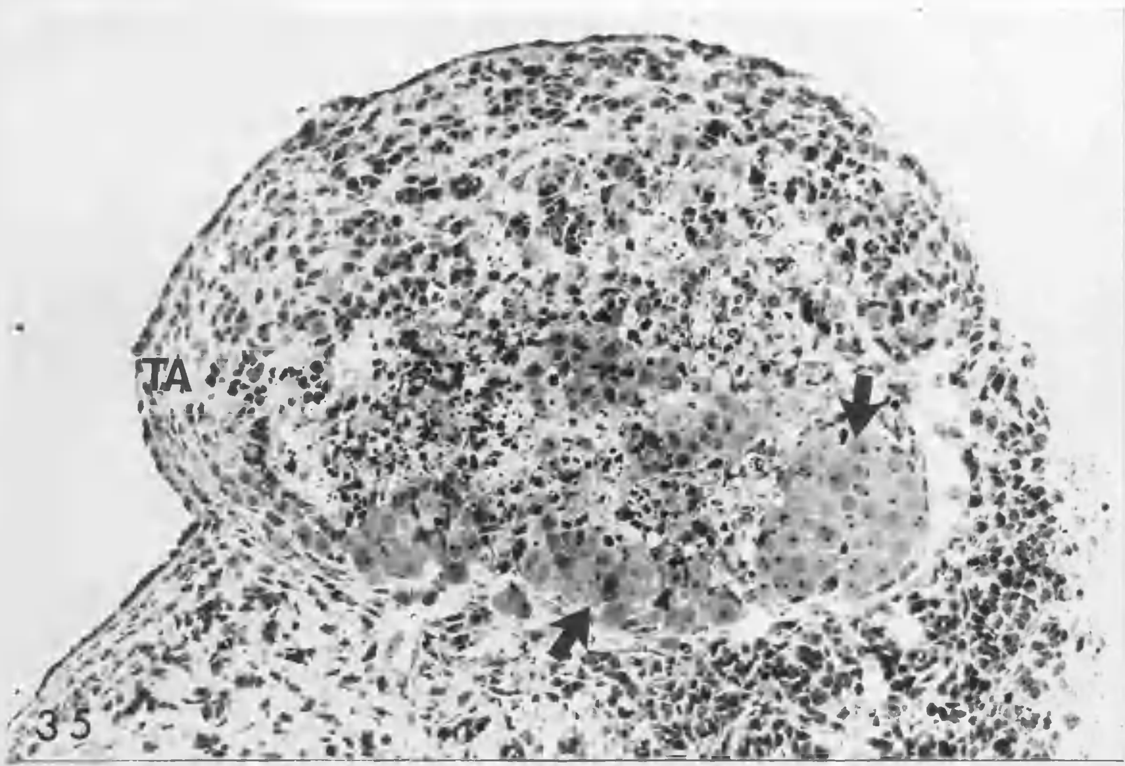
Experimental testis removed on 13.5dpc and cultured in 15-17dpc ovary-CM for 4 days showing disrupted testicular cords (arrows). A tunica albuginea is present at the testis edge (TA). (X250)

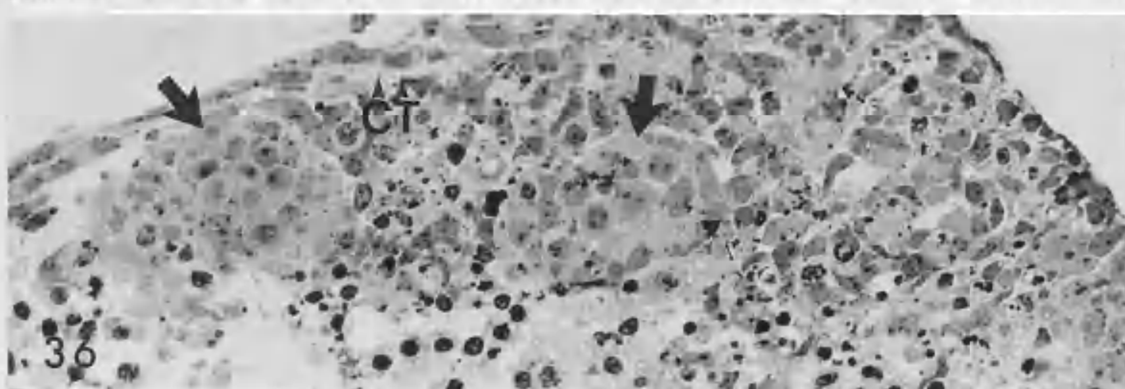
Figure 36

Control 13.5dpc testis cultured 4 days. Disrupted testicular cords are present near the edge of the tissue (arrows). Strands of connective tissue septa are evident within the interstitium (CT). (X500)

Figure 37

Isolated non-meiotic germ cell enveloped by thin cellular processes (arrows) within an experimental 13.5dpc testis cultured 4 days in 15-17dpc ovary-CM. (X7600)


TA
35


CT
36

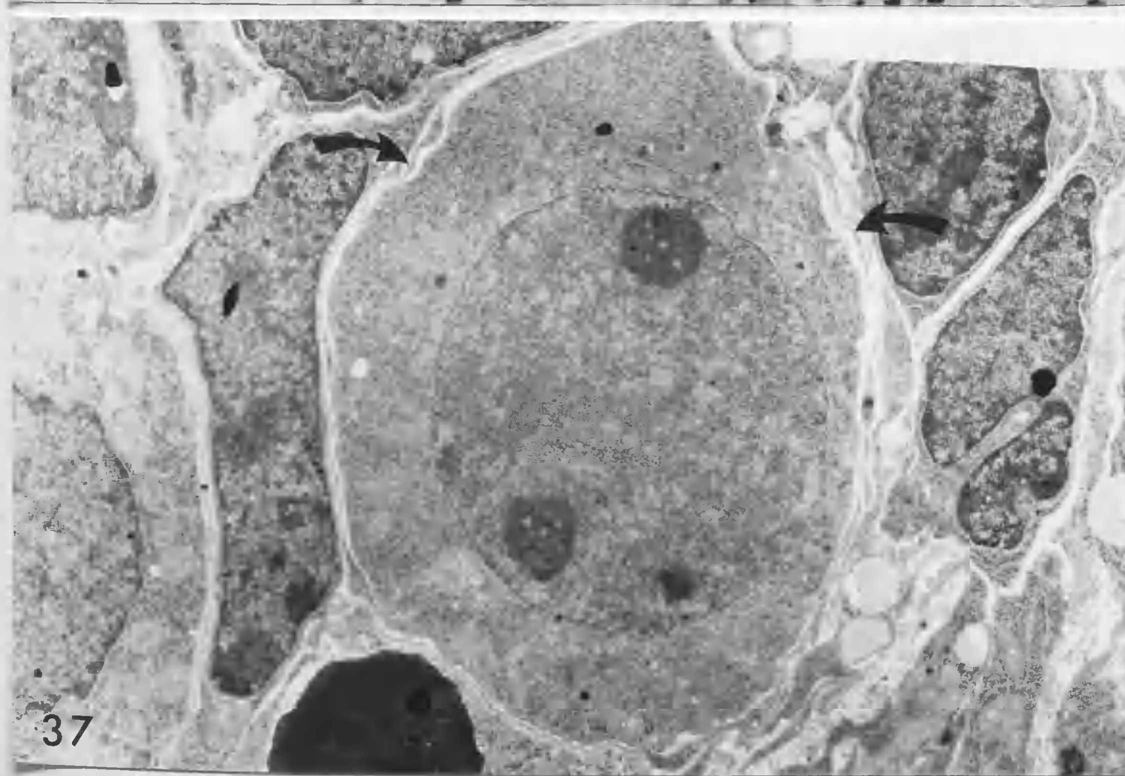
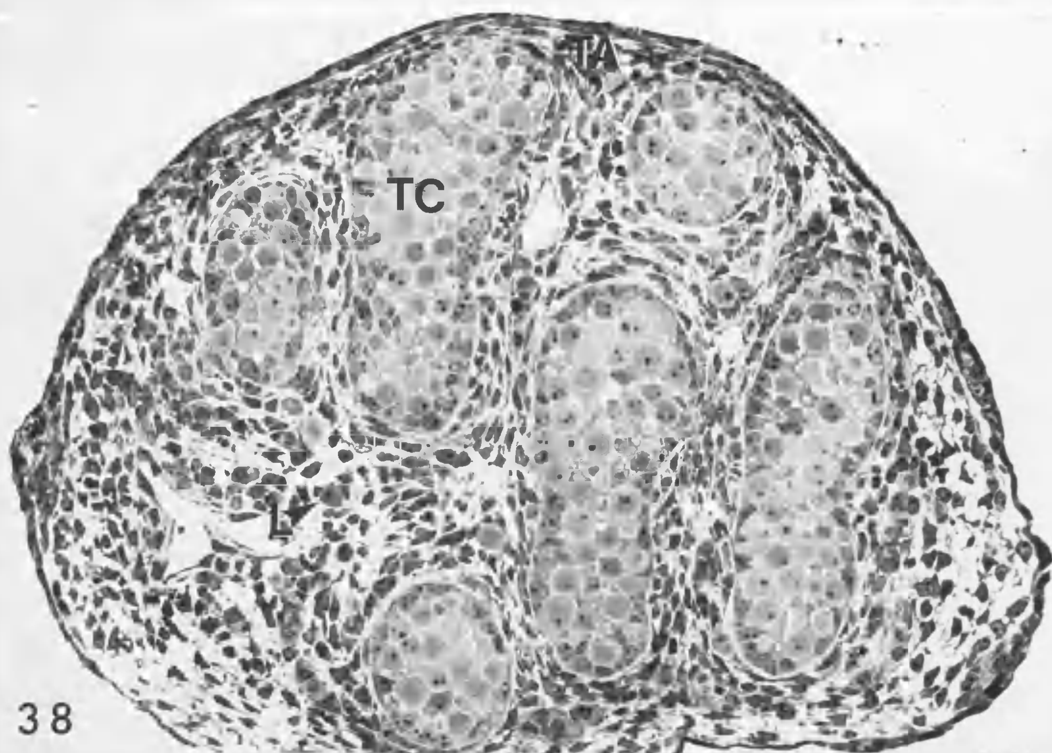

37

Figure 38

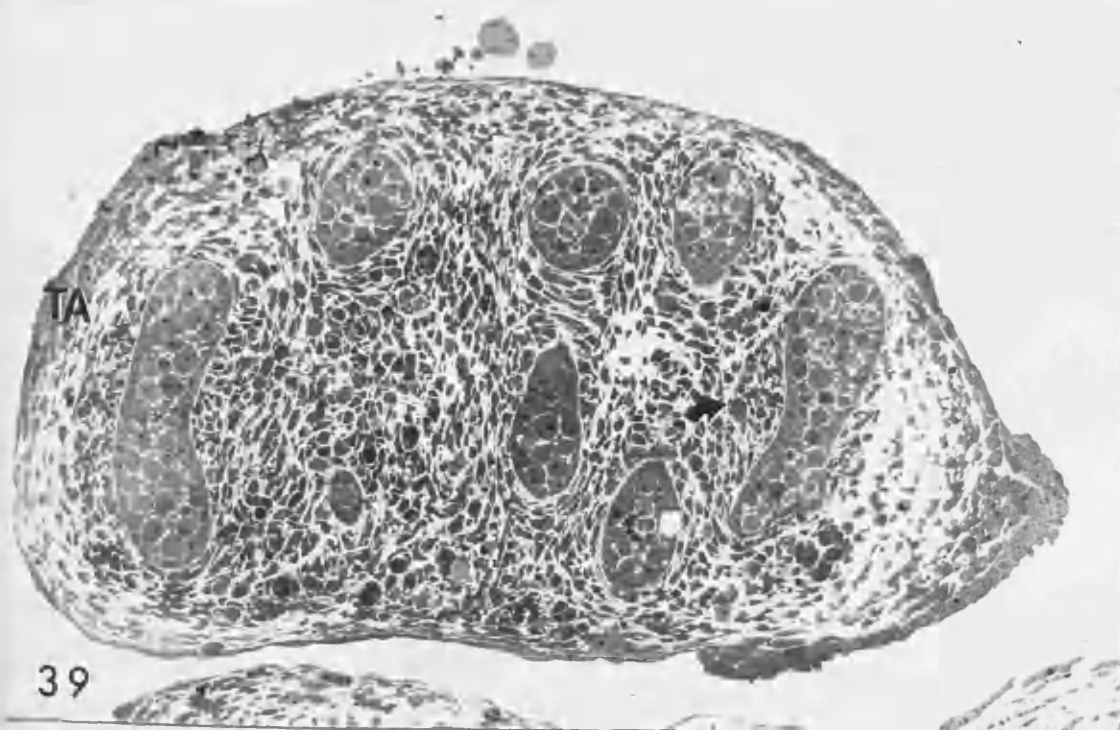
14dpc control testis cultured 4 days. Note tunica albuginea (TA), well-organised testicular cords (TC) with centrally placed germ cells and developing Leydig cells (L) within the interstitium. (X300)

Figure 39

Testis removed on 14dpc and cultured 4 days in 15-17dpc ovary-CM. Tunica albuginea is present (TA). Testicular cords appear reduced in size and interstitial tissue increased in volume. Differentiated Leydig cells (arrow) lie within the somatic tissue. (X200)



TA
TC
L
38



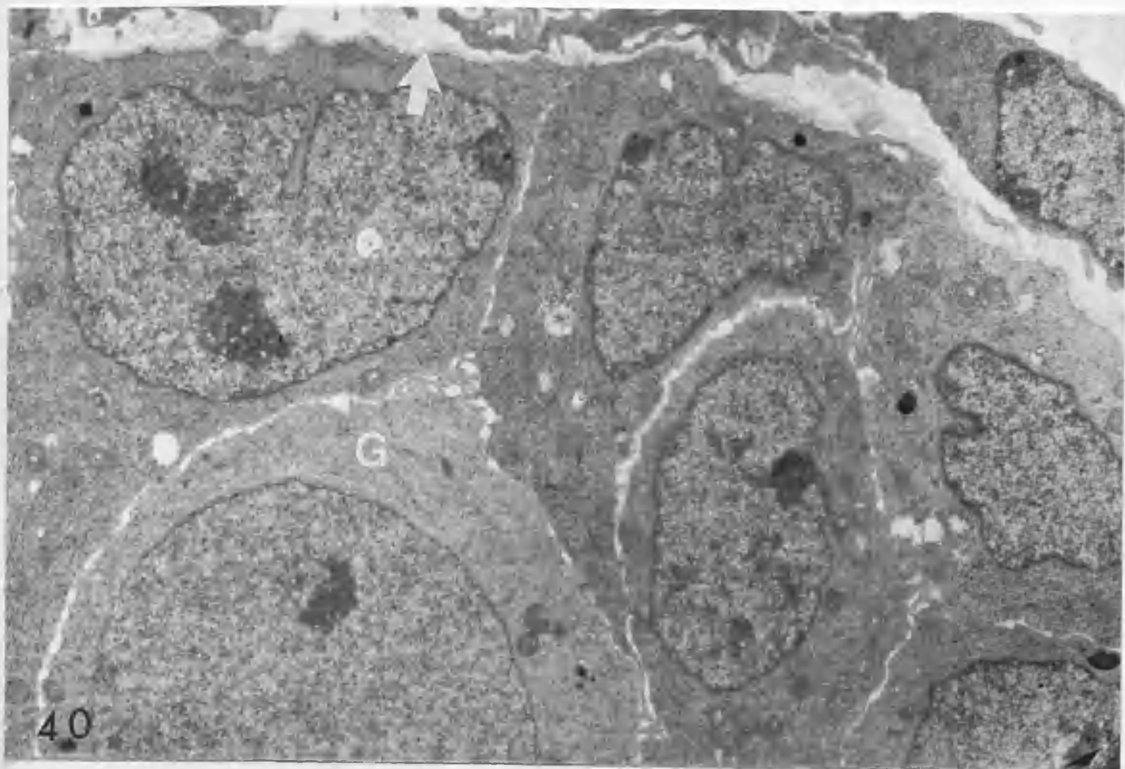
TA
39

Figure 40

Electron micrograph of experimental 14dpc testis cultured 4 days in 15-17dpc ovary-CM shows detail of testicular cord. Centrally located germ cell (G) shows no evidence of meiosis. A basal lamina (arrow) is present around the developing testicular cord. (X8500)

Figure 41

Electron micrograph of tunica albuginea at edge of experimental 14dpc testis cultured 4 days in 15-17dpc ovary-CM shows flattened somatic cells (arrows) and collagen fibres (CF). (X9000)


G
40

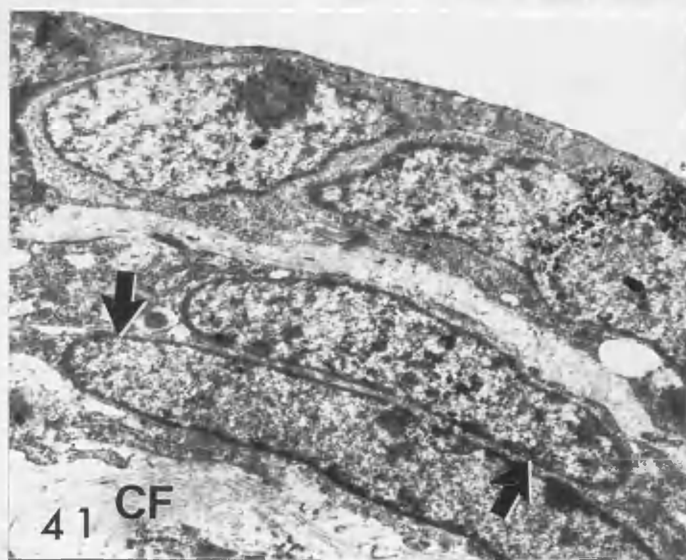

41
CF

Figure 42

Wolffian and Müllerian duct associated with one experimental 14dpc testis cultured 4 days in 15-17dpc ovary-CM. The Wolffian duct (W) is well-maintained whereas the adjacent Müllerian duct (M) shows signs of involution such as an epithelial cuff (arrows). (X800)

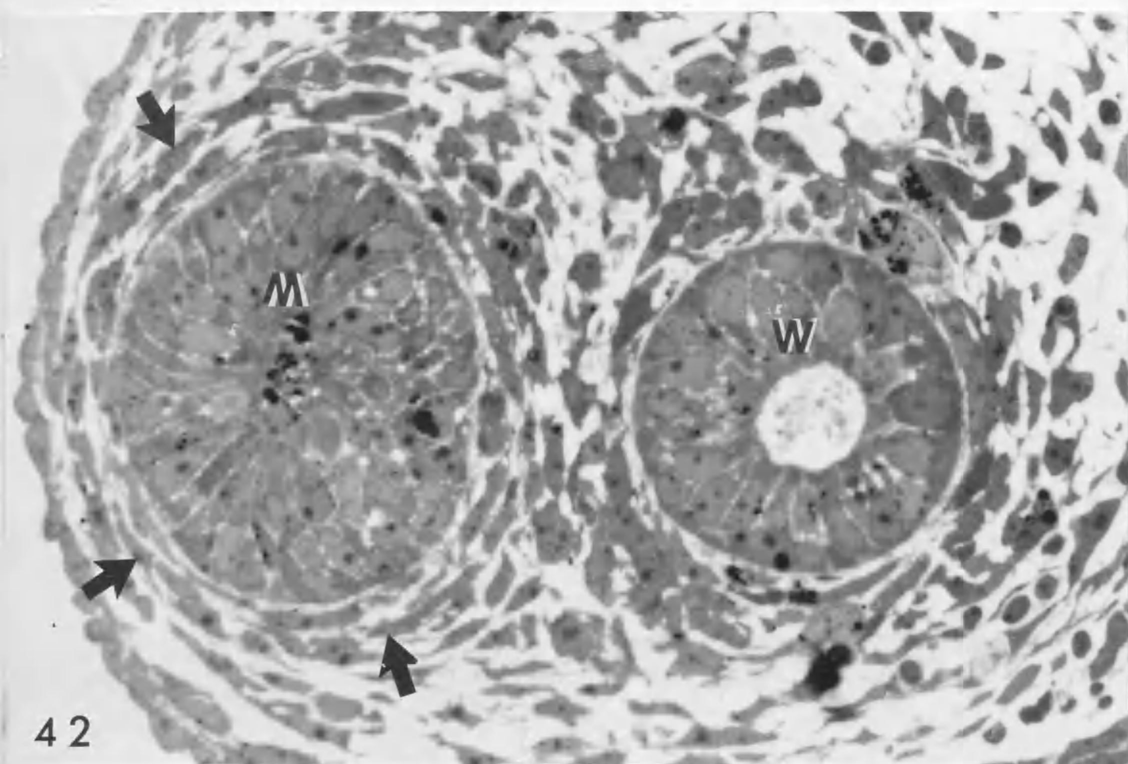

M
W
42

Figure 43

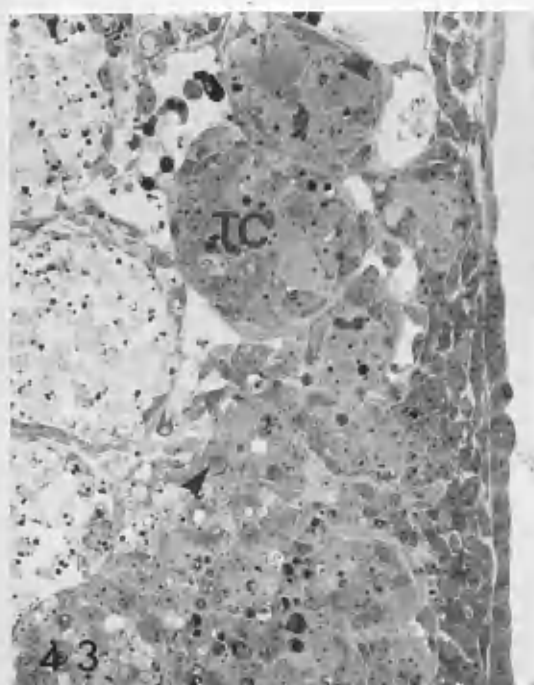
Control 12dpc testis cultured 2 days with one testis and one ovary. Testicular cords have developed (TC) surrounded by expanded blood vessels. Mitotic figures are evident both within cords and within the interstitial tissue (arrows). A developing tunica albuginea is present at testis edge. (X300)

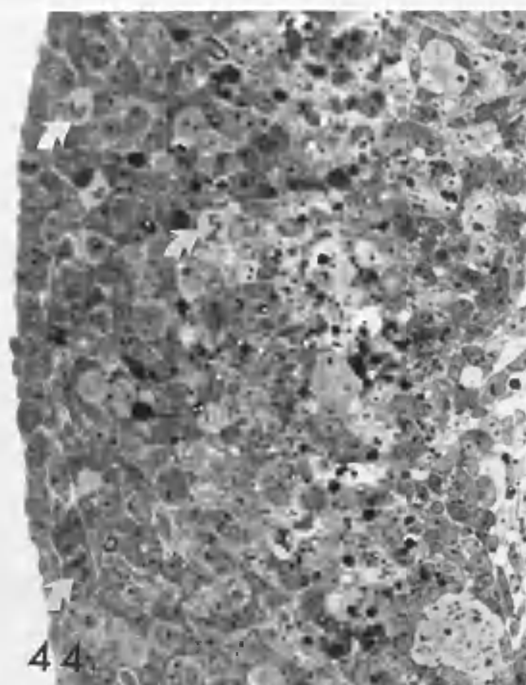
Figure 44

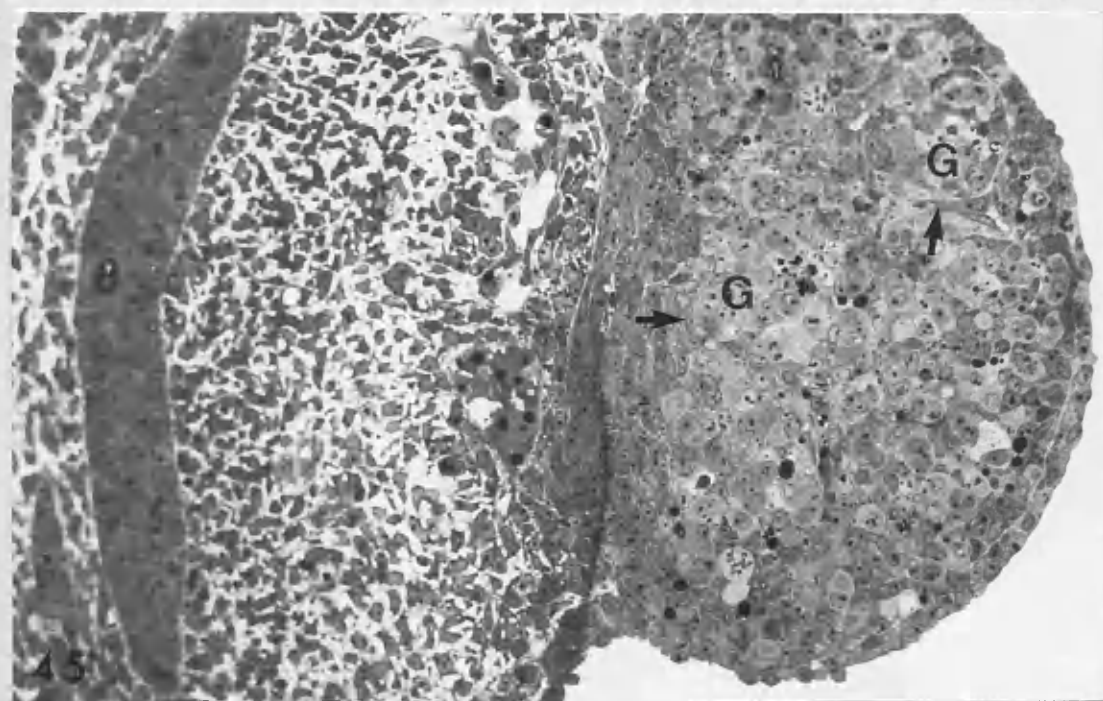
Experimental testis, removed on 12dpc and cultured for 2 days in 15-18dpc ovary-CM. Testicular cords have not developed. Numerous mitoses are evident throughout the tissue (arrows). (X300)

Figure 45

Experimental 12dpc ovary cultured 2 days in 15-18dpc ovary-CM with two testes. Germ cells are within indistinct ovigerous cords (G) outlined by flattened somatic cells (arrows). Mitotic figures are evident throughout the tissue. (X300)


TC
43


44


G
G
45

SECTION 3 - Adrenal gland-conditioned media

Zamboni and Upadhyay (1983) showed that ectopically located germ cells within the adrenal glands of the mouse, entered meiosis at the same time as those in the ovary regardless of sex, and continued along the meiotic pathway, in some cases forming structures resembling unilaminar follicles.

The present study was designed to determine the effect of adrenal CM on the development of fetal testes and ovaries.

RESULTS

Two different conditioned media were used to culture 13dpc ovaries and testes for either 2 or 4 days.

Table 7

Adrenal gland-conditioned media

Expt No.	Sex	Adrenal 14-16*	CM(dpc) 14-17**	Duration of culture (days)	Total No. paired explants cultured***
3.1	F(1)	X		2	10
	F	X		4	2
	F		X	4	6
3.2	M(1)	X		2	4
	M	X		4	4
	M		X	4	10

* 14-16dpc adrenal CM generated by 8 male and 4 female adrenal glands in 2.5mls standard medium (with 10% FCS).

** 14-17dpc adrenal CM generated by 8 male and 4 female adrenal glands in 0.8mls standard medium (with 10% FCS).

***Controls consisted of 13dpc testes and ovaries cultured in standard medium for 2 and 4 days.

1. Gonads were pre-incubated in 0.01% EDTA for 30mins prior to culture in CM.

All experiments were examined with the transmission electron microscope.

3.1 Effects on ovarian development

Ten ovaries, excised on 13dpc were cultured for 2 days in 14-16dpc adrenal-CM. Ovaries contained large numbers of germ cells which had continued mitosis _in vitro_ (Fig. 46) and resembled control ovaries. In addition, ovaries had increased in size and were more rounded in appearance than the 13dpc ovary. Small blood vessels permeated the interstitial tissue and connective tissue septa had developed, outlining the germ cells arranged within ovigerous cords. Electron microscopic examination revealed that some follicular cells had extended processes in between adjacent germ cells (Fig. 47). In both culture conditions, mitotic figures were occasionally seen and there was little sign of necrosis.

In all ovaries, the Müllerian duct was consistently larger than the Wolffian, with mitoses occasionally seen.

Those ovaries (2 pairs) cultured for 2 days longer were also well-maintained (Fig. 48). Connective tissue septa were more evident than in cultured controls, with thick collagenous fibres extending between ovigerous cords, indicating that ovarian organisation had continued. However, ovigerous cords were not as well developed as the equivalent _in vivo_ ovary (17dpc).

Synaptonemal complexes were present within oocyte nuclei indicating that the zygotene stage of meiosis had been reached, although mitotic figures were also present.

Four day culture of 13dpc ovaries in 14-17dpc

concentrated adrenal CM (i.e. cultured in a smaller volume of medium) resulted in a significant advance in meiosis with numerous synaptonemal complexes within oocyte nuclei (Fig. 49; Table 8). The data shown in Table 8 is derived from counts of synaptonemal complexes within a total of 34 control and 34 experimental oocytes sampled from 6 and 4 ovaries respectively. Cultured control ovaries contained fewer complexes (Fig. 50). Ovigerous cords were similar to those within ovaries cultured for an equivalent period in 14-16dpc adrenal CM.

In conclusion, adrenal CM had a beneficial effect on ovarian development. In addition, concentrated 14-17dpc adrenal CM significantly promoted meiosis as compared to both controls cultured in standard medium and equivalent ovaries cultured in 14-16dpc adrenal-CM.

Table 8

Average number of synaptonemal complexes per oocyte per ovary in 6 control 13dpc ovaries cultured in standard medium and 4 experimental 13dpc ovaries cultured in 14-17dpc adrenal-CM.

	AVERAGE NUMBER OF SYNAPTONEMAL COMPLEXES/OOCYTE	
OVARY	CONTROLS	EXPERIMENTALS
1	0.33	5.4
2	0.83	5.9
3	0.78	15.5
4	0.00	6.8
5	0.33	
6	1.5	
MEAN (± SEM)	**0.63 ± 0.28**	**8.4 ± 2.74***

* $t=4.08$, $df=8$, $P < 0.004$ using Student's t-test (2-tailed).

3.2 Effects on testicular development

Sexual differentiation of the four 13dpc testes proceeded within 14-16dpc adrenal CM after 2 days in vitro. Testicular cords were larger and more rounded that at the time of excision. However, cords were also more irregular in outline and the normal arrangement of pre-Sertoli cells peripherally and central germ cells was disorganised (Fig. 51). Cultured controls were more similar to the in vivo 15dpc testis (Fig. 52).

Electron microscopy of experimental testes revealed that a basal lamina had developed at the periphery of testicular cords, and that germ cells were not confined to central locations (Fig. 53). Flattened cells were closely associated with most of the surface of cords.

Tunica albuginea development was also affected in experimental testes, although there were some flattened cells underlying the surface epithelium in places. Blood vessels within the interstitial tissue had expanded lumens and connective tissue strands extended throughout testes.

Two days further culture of four 13dpc testes resulted in the complete breakdown of cord structure in both controls and those cultured in CM. In one experimental testis (Fig. 54), a large clump of germ cells and pre-Sertoli cells aggregated with necrotic tissue lying centrally. As necrosis was evident mainly in the depths of both control (Fig. 55) and experimental tissue, this was again taken to be a size effect rather than an induced

effect. Flattened cells outlined part of the 'cord'. Other testes contained small clumps of germ cells which lay in the deeper aspects of the gonad, unlike in ovaries where germ cells are present throughout the tissue. Tunica albuginea formation was absent in 4 out of 5 experimental testes, and was not as well developed as in the testis at 17dpc. There was no indication of early entry into meiosis of germ cells within disrupted cords or isolated within the somatic tissue (Fig. 56).

Ten 13dpc testes were also cultured in concentrated 14-17dpc adrenal-CM for 4 days. At the end of the culture period they contained necrotic centres although cords were more degenerated than when cultured for an equivalent period in 14-16dpc adrenal CM (Fig. 57). Few germ cells were present and these had not entered meiosis. A tunica albuginea was present in all testes, underlying the flattened surface epithelium.

In conclusion, meiosis was not induced in testes cultured in adrenal CM. Development did not continue after 2 days culture in both control and conditioned medium, although a tunica albuginea developed in those testes cultured for 4 days in 14-17dpc CM, which was only present in one out of 5 testes cultured in 14-16dpc adrenal CM for an equivalent period.

Figure 46

13dpc ovary cultured 2 days in 14-16dpc adrenal-CM contains several oocytes arranged in ovigerous cords (arrows). (X700)

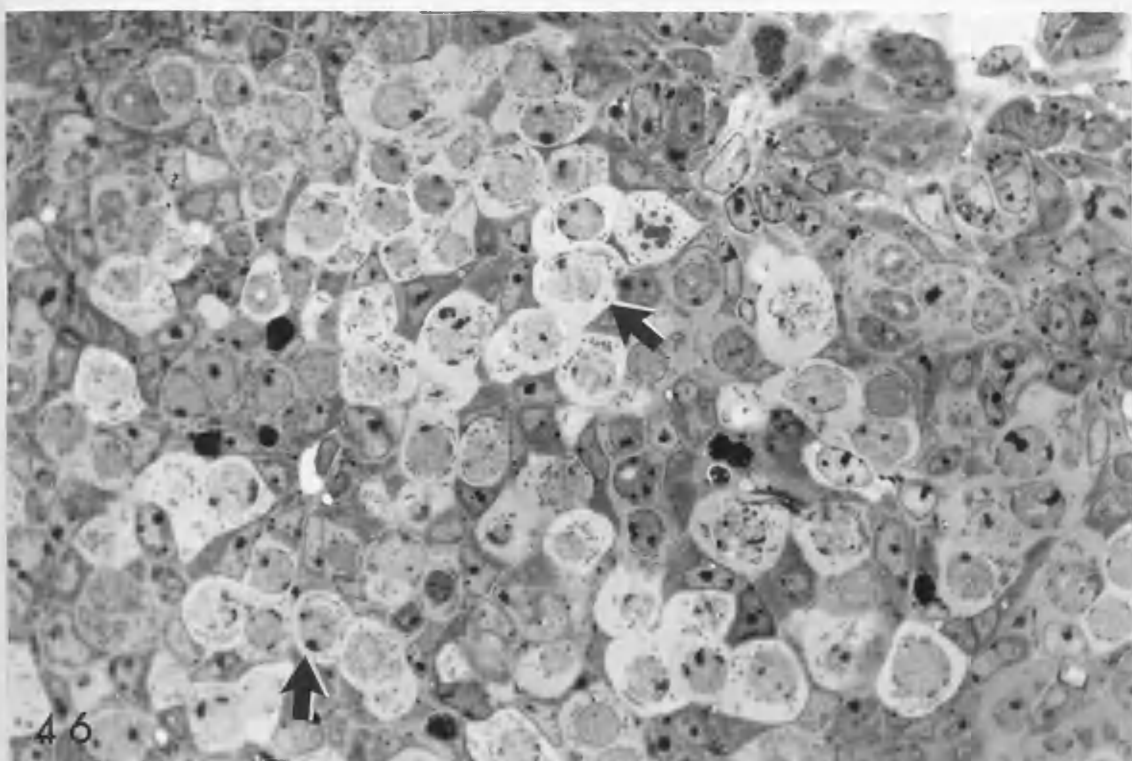

46

Figure 47

Electron micrograph of ovigerous cord within a 13dpc ovary cultured 2 days in 14-16dpc adrenal-CM. Follicular cell processes (arrows) begin to separate the oocytes. (X3400)

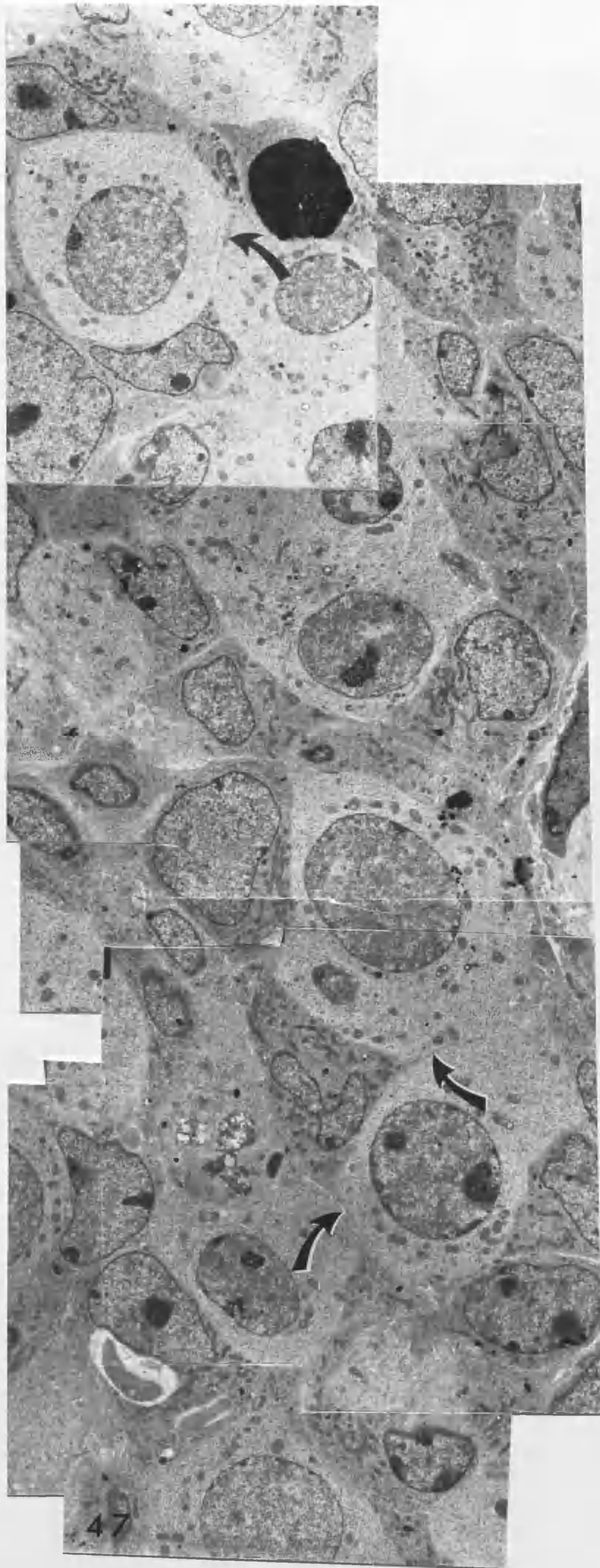
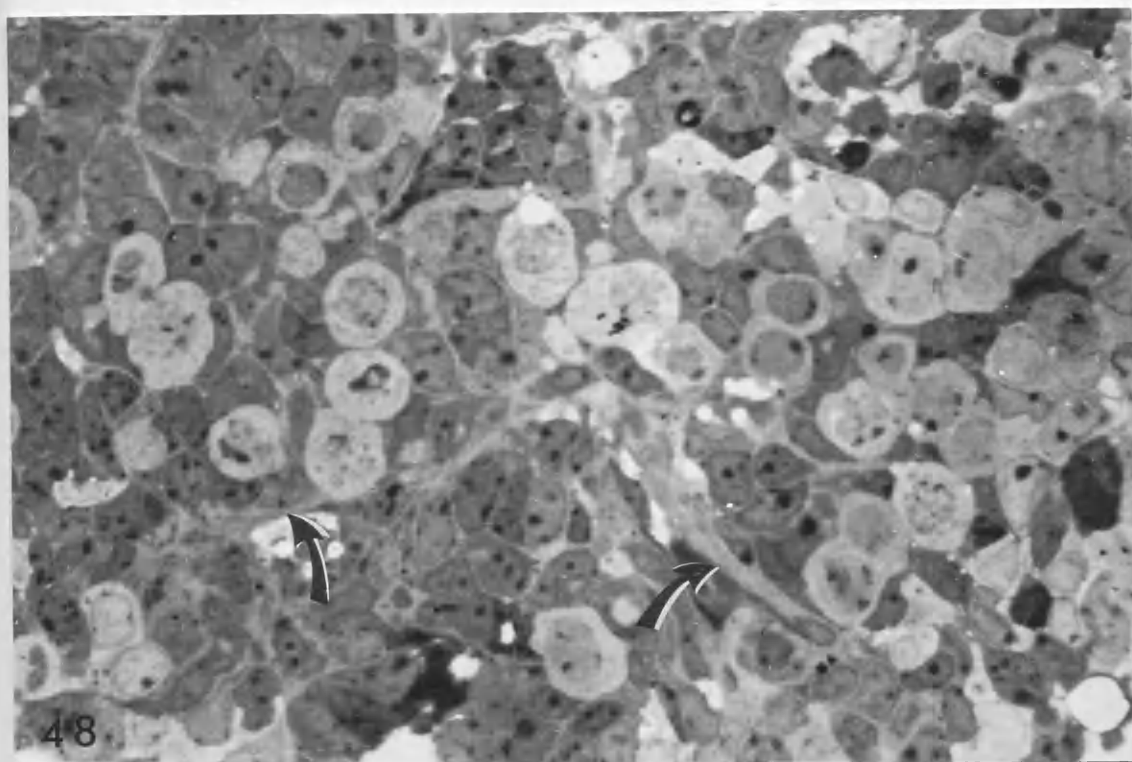

47

Figure 48

Experimental 13dpc ovary cultured 4 days in 14-16dpc adrenal-CM. Connective tissue septa (arrows) are evident throughout the somatic tissue. Oocytes are arranged within developing ovigerous cords. (X700)

Figure 49

Electron micrograph of oocyte within 13dpc ovary cultured 4 days in 14-17dpc adrenal-CM. Several synaptonemal complexes (arrows) indicate that the pachytene stage of meiotic prophase has been reached. (X11200)


48

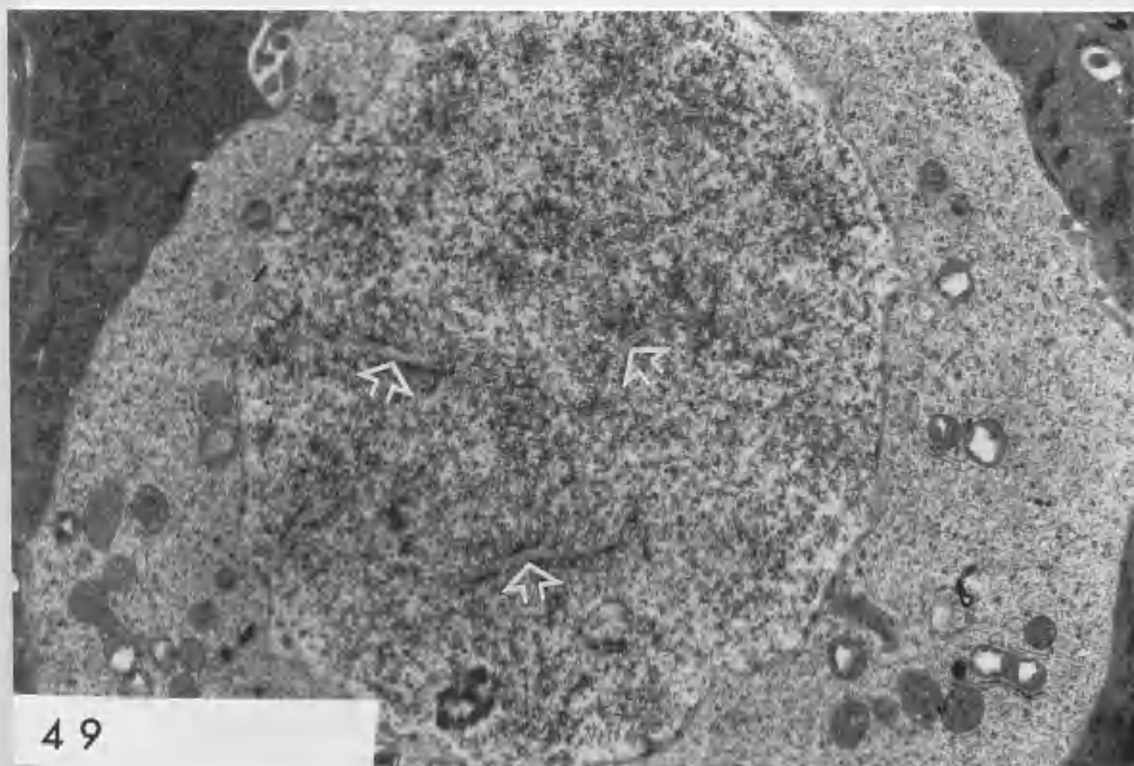

49

Figure 50

Control 13dpc ovary cultured 4 days. Fine follicular cell processes (arrows) separate 2 oocytes containing synaptonemal complexes (SC). (X2000)

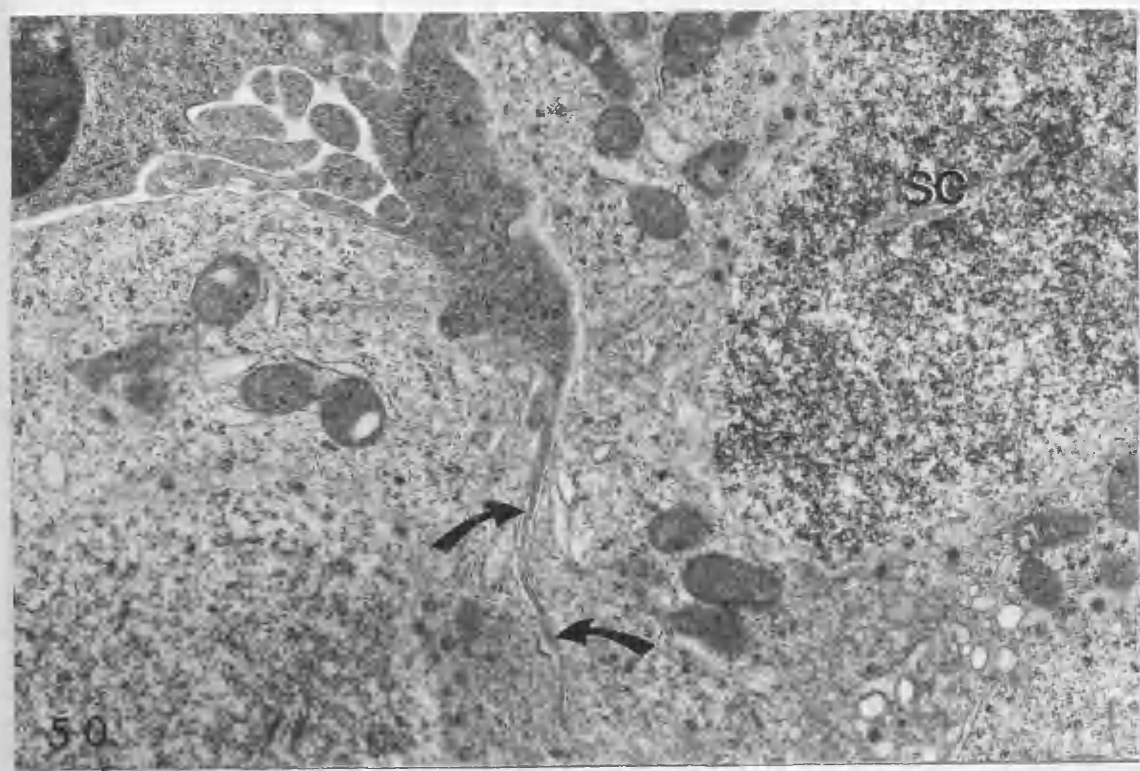

SC
50

Figure 51

Experimental 13dpc testis cultured 2 days in 14-16dpc adrenal-CM showing testicular cord (TC). Normal arrangement of central germ cells and peripheral pre-Sertoli cells is disorganised. (X500)

Figure 52

13dpc control testis cultured 2 days. Testicular cords (TC) are well-organised. Connective tissue septa are evident within the interstitial tissue (arrow). (X500)

Figure 53

Electron micrograph of experimental 13dpc testis cultured 2 days in 14-16dpc adrenal-CM. Pre-Sertoli cells (S) are displaced centrally by germ cells (G). A basal lamina is evident at cord edge (arrow). (X5000)

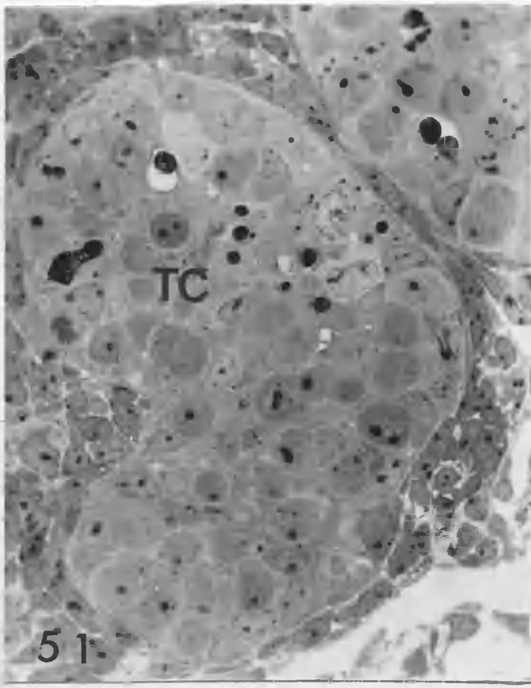
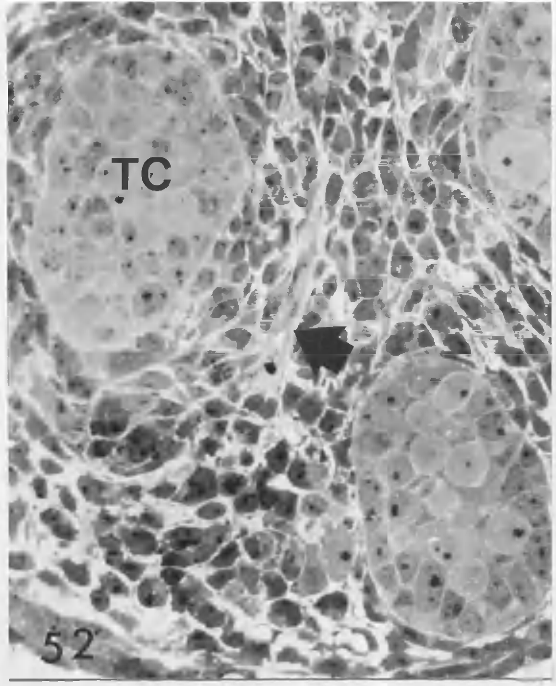
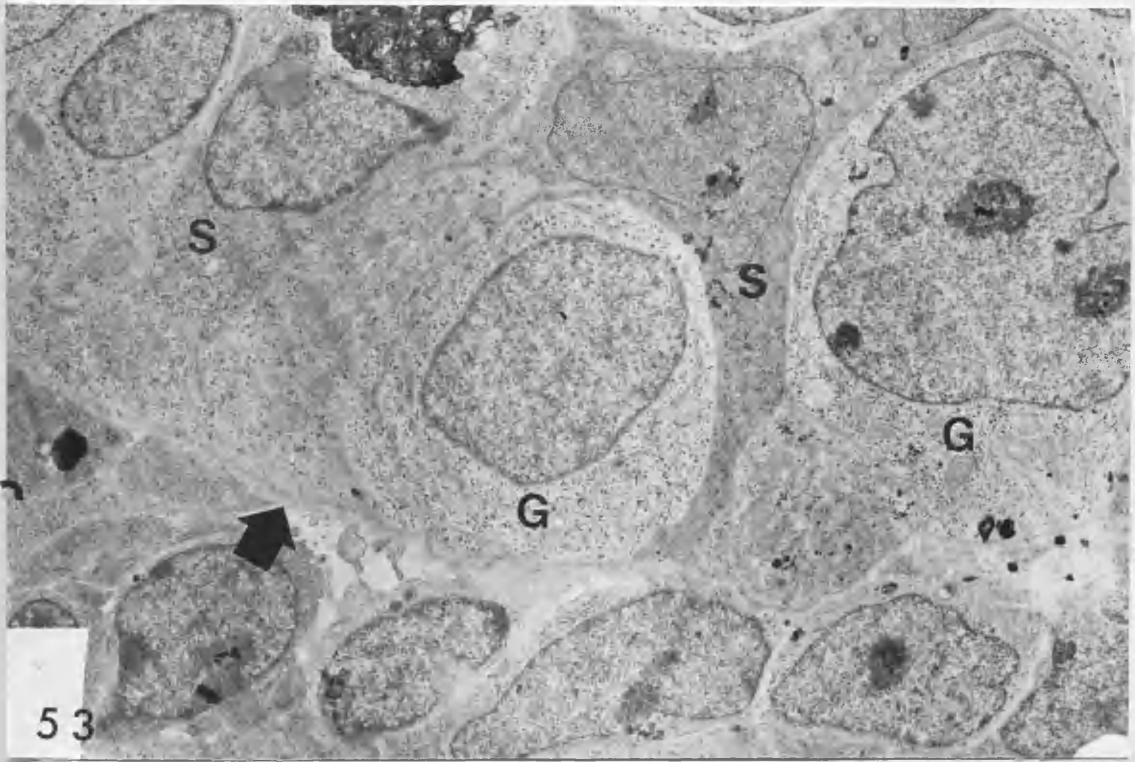
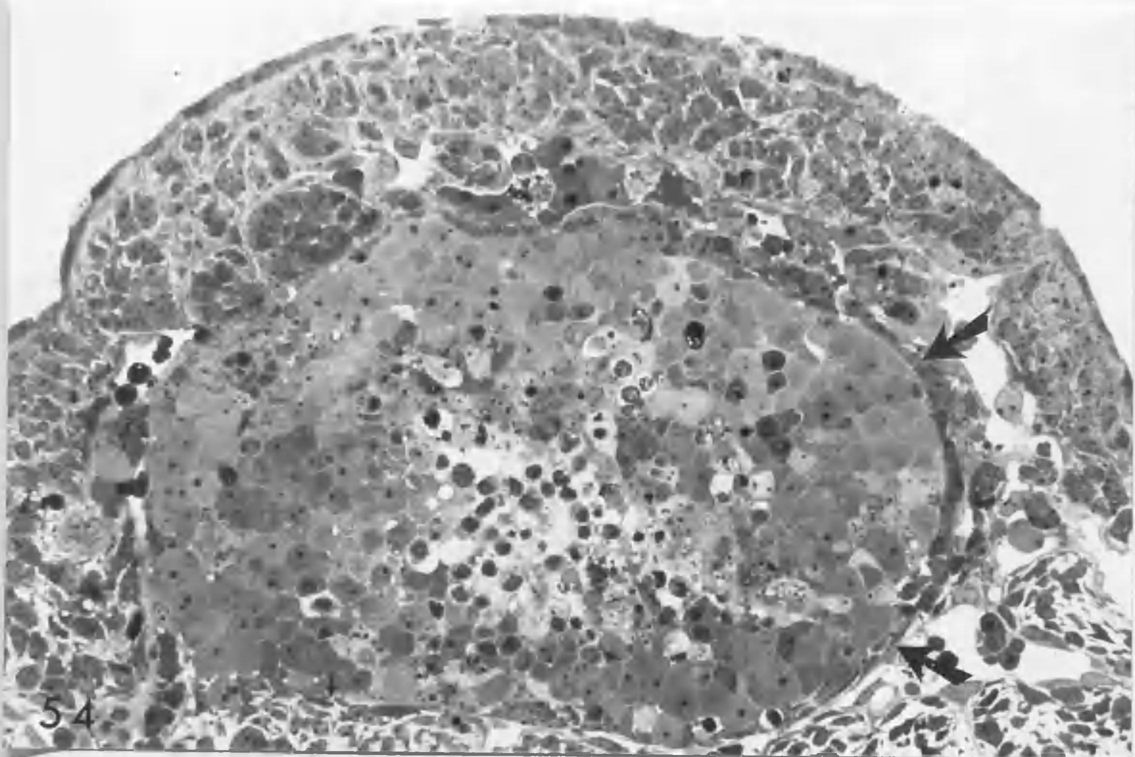

TC
51

TC
52

S
S
G
G
G
53

Figure 54

Experimental 13dpc testis cultured 4 days in 14-16dpc adrenal-CM. Individual testicular cords have broken down and a large 'cord' has formed outlined by flattened somatic cells (arrows). (X450)

Figure 55

Control 13dpc testis cultured 4 days. Testicular cords are disrupted. A group of germ cells (arrow) is surrounded by expanded blood vessels. There was no sign of meiosis within disrupted cords or isolated germ cells (arrow-head). (X500)


54

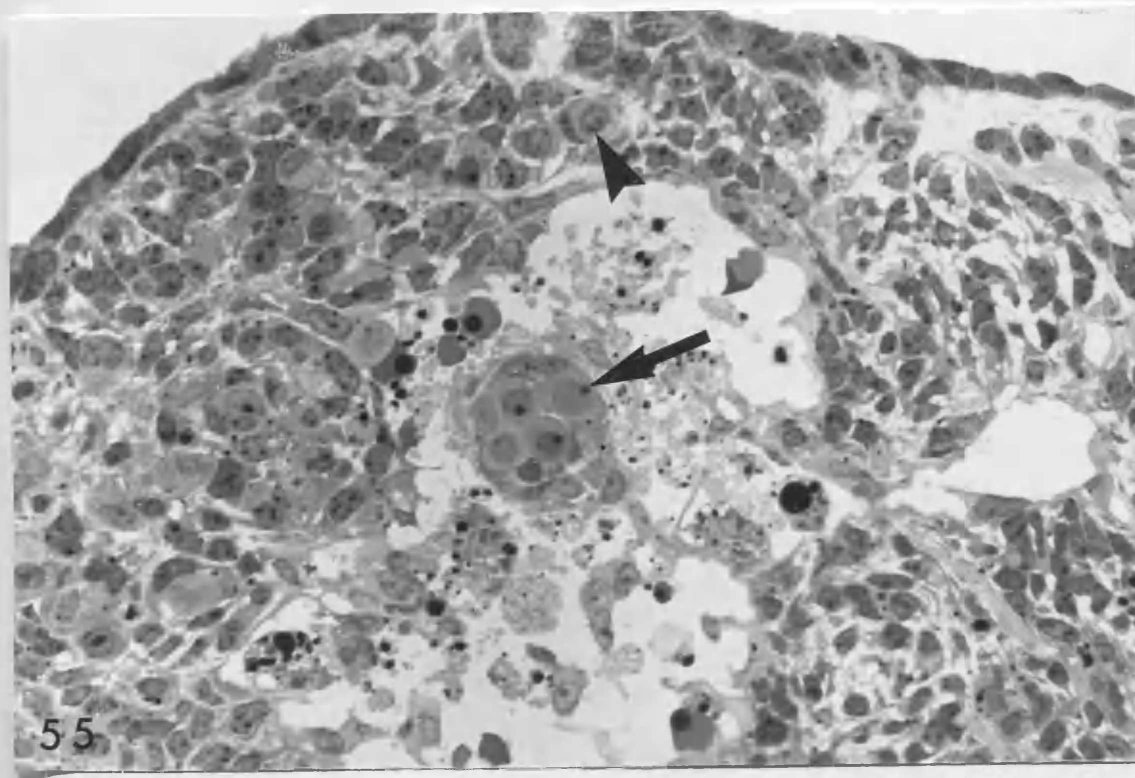
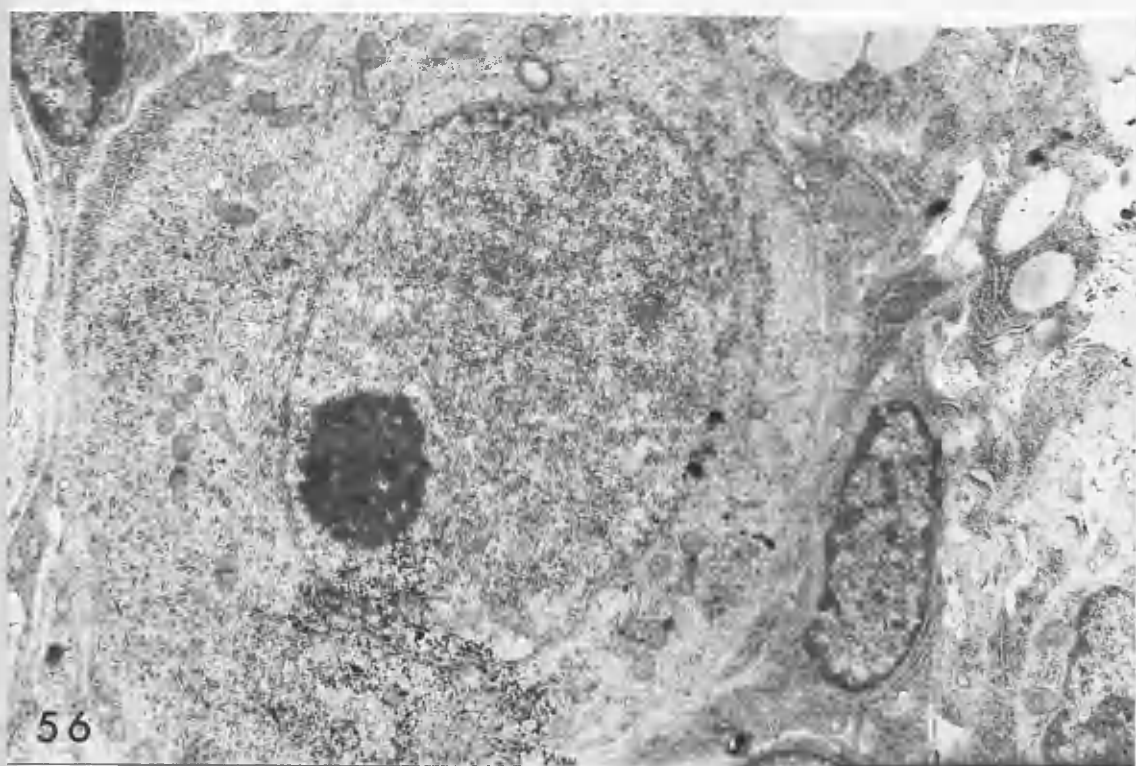

55

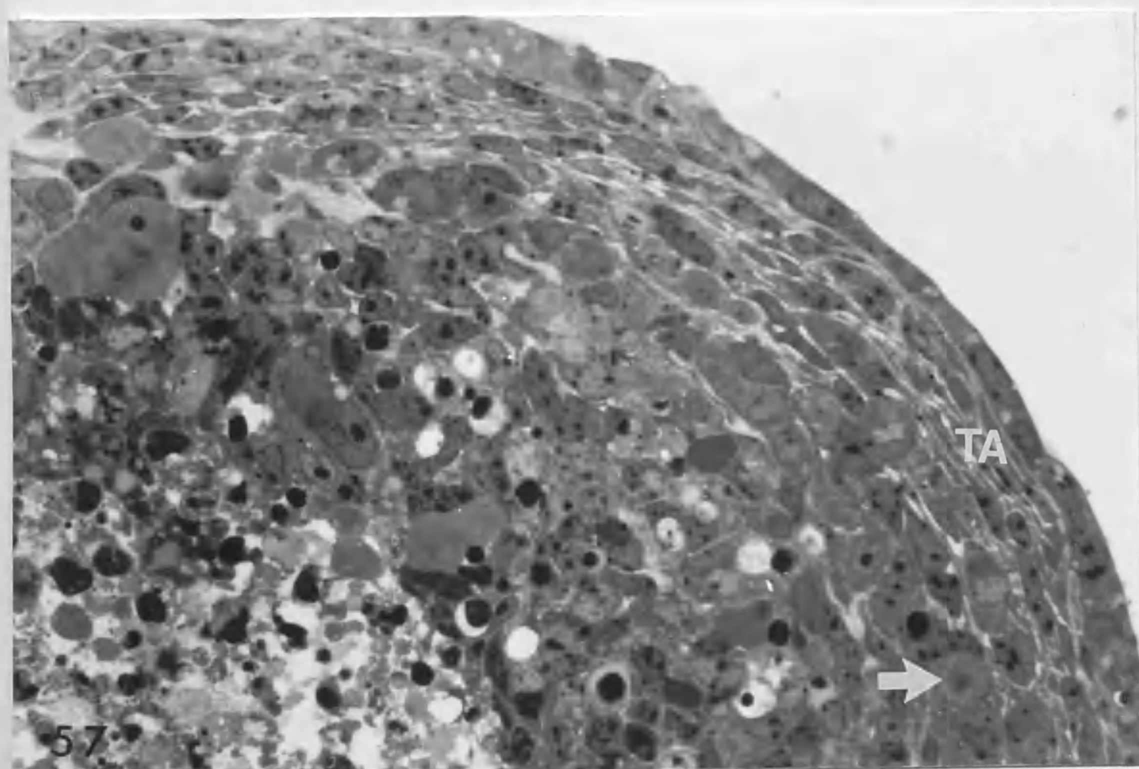
Figure 56

Electron micrograph of an isolated germ cell within a 13dpc testis cultured 4 days in 14-16dpc adrenal-CM shows no sign of early entry into meiosis. (X11000)

Figure 57

13dpc testis cultured 4 days in 14-17dpc adrenal-CM. A tunica albuginea is present (TA). Testicular cord structure has broken down and isolated germ cells are evident (arrow). (X600)


56


TA
57

SECTION 4 - Isolated germ cell cultures

<u>Histochemical analysis</u>:- Both techniques used positively identified germ cells both within isolated cultures and control cultures. However, on some occasions no germ cells or somatic cells were found after staining. It was concluded that these were washed off as a result of immersion within reagent. Both techniques were considered reliable, but on balance, the red PGCs (Fig. 58) produced by the Barka and Anderson (1962) technique were more distinctive than the darker tetrazolium stained cells.

<u>12.5dpc female germ cells in 14-15dpc ovary-CM for 7 days</u>

Germ cells were isolated from 12 ovaries excised on 12.5dpc and cultured for one day. Following culture for one day in CM, germ cells were mainly floating singly or in small clumps of 3-4 cells within the CM. Somatic cells were also present in small clumps or single. By day 4 the majority of cells had settled on the fibronectin-coated substrate (Fig. 59), and 2 days later these were very well attached (this was shown when a glass micropipette was required to dislodge germ cells). At the end of the culture period germ cell number appeared reduced although viability was high (73%).

Histochemistry positively identified germ cells both within the isolated culture and within the residual ovarian tissue control culture, showing that many germ cells remained within the ovaries at the end of the 8 day culture

period.

12.5dpc male germ cells in 14-15dpc testis-CM for 7 days

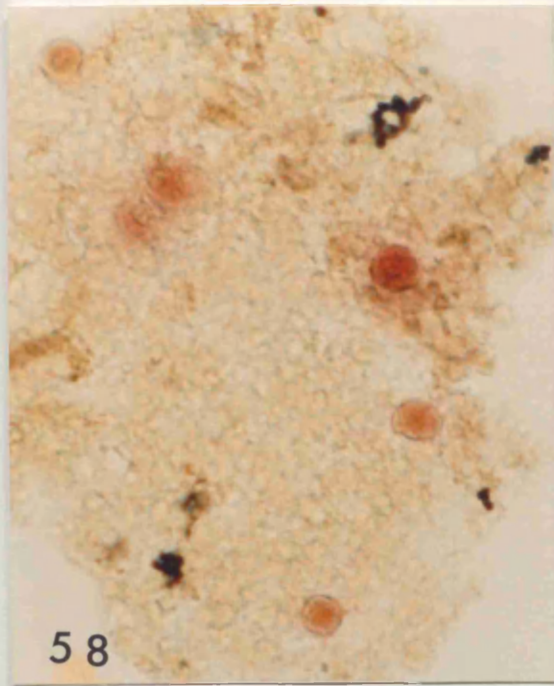
Cultures resembled equivalent female germ cells throughout the period of culture and viability was also high at the end of the 7 day culture period (84%), although again the number of germ cells was reduced. Positive identification of germ cells was also carried out histochemically.

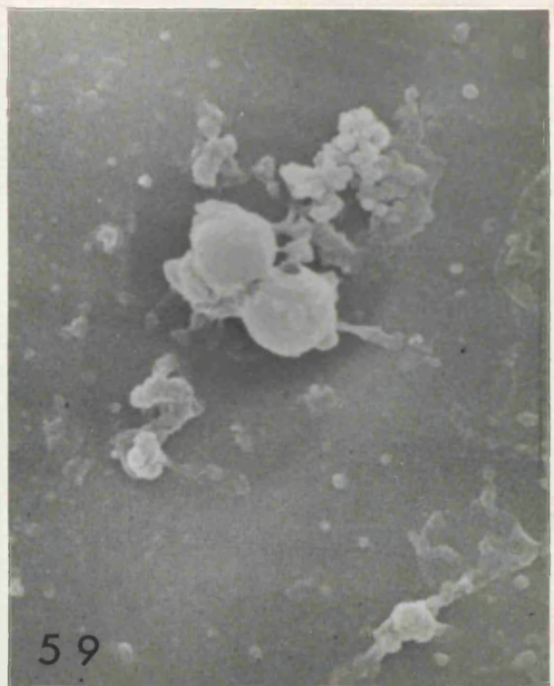
Further experiments were performed combining germ cells from gonads excised on 11.5, 12 and 13dpc in with 14-15dpc testis- and ovary-CM for 2-7 days. Throughout the culture period cultures appeared similar to those described above with no apparent differences between male and female germ cells, whether cultured within CM of the same or of the opposite sex. Viabilities ranged from 45% after 2 days to 60-66% after 4 days.

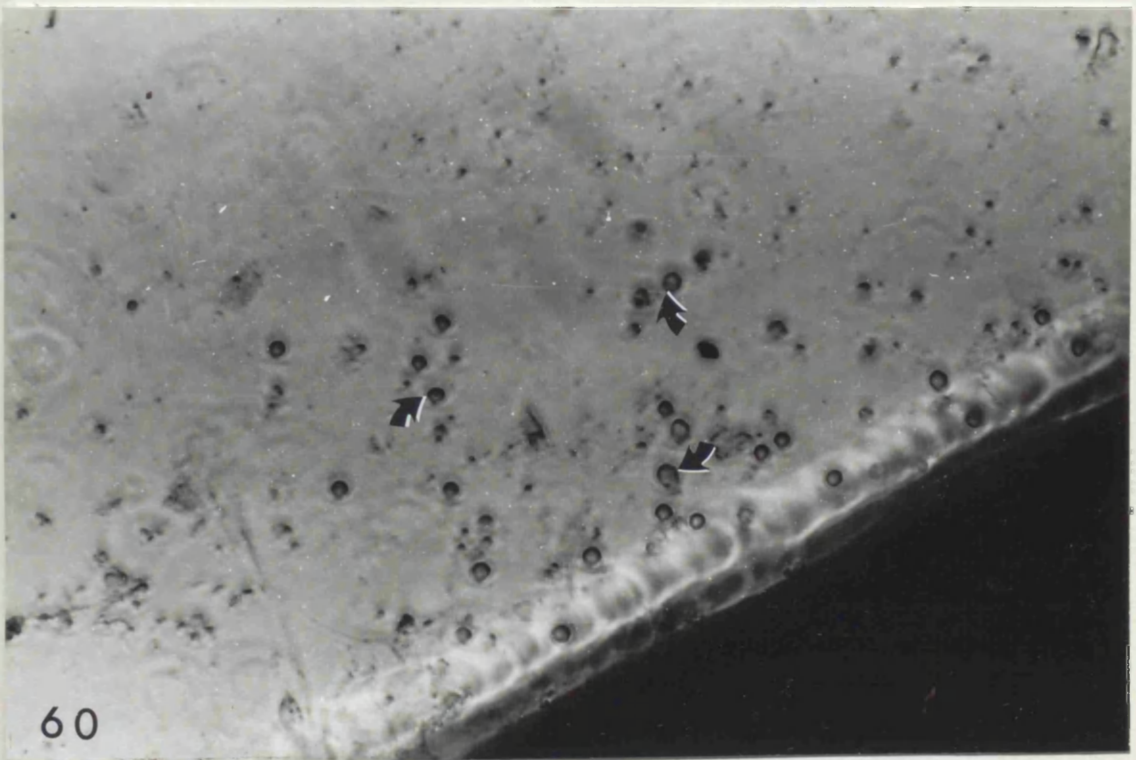
Transmission electron microscopy :- Following embedding in resin, coverslips were sectioned for analysis under the light and transmission electron microscope. However, although the coverslips cut easily, no germ cells were found after staining with toluidine blue. It is probable that cells were washed off during processing.

Scanning electron microscopy(SEM) :- Similar problems were encountered in processing for SEM. A pair of cells which may be germ cells is shown in Fig. 60. Conclusive

identification of germ cells is impossible without the use of a germ cell marker. Staining for alkaline phosphatase prior to processing was attempted, however, no germ cells remained.


58


59


60

SECTION 5 - Analysis of conditioned media

Characterisation of diffusible fetal gonadal factors was carried out using native PAGE gel electrophoresis. Several batches of conditioned media, generated from varying stages of development, were run. However, due to the small amounts of protein present, bands were often faint.

The sensitivity range of the silver stain used was 1-5ng of each protein/μl. The Coomassie stain had a lower sensitivity of 20-30ng of each protein/μl. It was found that this stain alone was insufficient and gels were subsequently stained with silver. The standard proteins of known molecular weights give distinct and deep staining bands with both stains in the molecular weight range 67Kda-669Kda.

Examination of bands in 13-15dpc testis-CM as compared to 13-15dpc ovary-CM revealed that many more bands were present (Fig. 61). In addition these tended to be of a higher molecular weight than those produced by equivalent ovary-CM. Consistent bands were noted in 4 samples of 13-15dpc testis-CM in the molecular weight ranges of 540-560Kda and 420-440Kda. Bands in both of these regions were also present in 1 out 3 samples of 13-15dpc ovary-CM and in 13-17dpc and 15-16dpc ovary-CM, and in 17-19dpc testis- and ovary-CM. Other bands exclusive to 13-15dpc testis-CM were in the molecular weight ranges of 465-485KDa, 310-360KDa, and 210-240KDa. Protein bands

between 115KDa and 132KDa were also noted. Bands unique to 13-15dpc ovary-CM were all below 130KDa.

Conditioned medium generated from ovaries excised on 13dpc and cultured for 4 days was also analysed (Fig. 61). Following two more days development in vitro, bands ranging from approximately 67KDa to 240KDa appeared.

Analysis of 17-19dpc testis- and ovary-CM showed that bands of a lower molecular weight range were present within the testis-CM, ranging from 45KDa to approximately 440KDa, with a distinct band in the region of 140KDa (Fig. 62). These media proved highly concentrated as gonads are considerably larger at this stage and more gonads were used to generate the CM. As a result, streaking occurred which obscured discrete bands.

Protein analysis

Results of protein analysis were inconsistent and it is concluded that a more sensitive method must be used in future, suitable for picomolar quantities of soluble protein.

Conditioned media generated from 13dpc ovaries and testes cultured for one day (an average of one gonad per ml) contained approximately 30μg/ml and 23μg/ml of protein, respectively. Testis-CM generated from 13-15dpc (an average of 12 gonads per ml) gave values of -8 to -1 μg/ml following filtration, and -2μg/ml without filtration. This suggests that filtering CM does not remove proteins.

However, ovary-CM generated similarly contained -1μg of protein per ml after filtration, but 6μg/ml in non-filtered CM. Analysis of one sample 14-15dpc ovary-CM indicated that approximately 15μg/ml (an average of 5 gonads per ml) of protein was present, although a similar sample apparentely contained around -7μg/ml. Therefore, no conclusions can be drawn from this data.

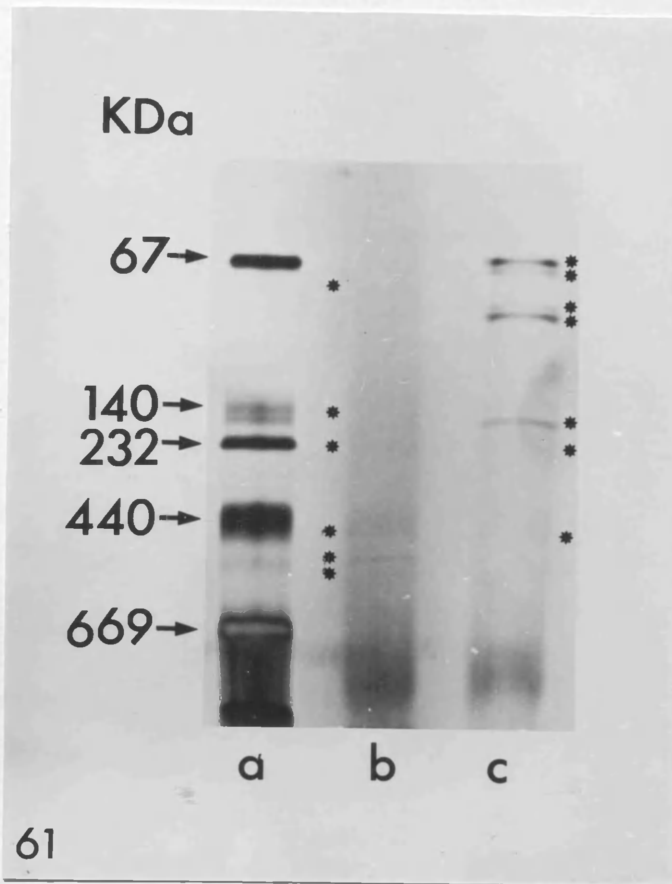
Adrenal conditioned media, containing 10% FCS, were also subjected to protein analysis in order to investigate the effects of filtration on serum-containing media. Results were more consistent, probably because of the high protein content of the FCS. There was a distinct reduction in protein content in CM that was both centrifuged and filtered (approximately 156μg/ml) as compared to the same medium which had been spun only (193μg/ml).

Figure 61

Lane (a) standard proteins Thyroglobulin (669kDa), Ferritin (440kDa), Catalase (232kDa), Lactase (140kDa), Albumin (67kDa). Lane (b) 13-15dpc testis-CM. Lane (c) 13-17dpc ovary-CM.

Figure 62

Lane (a) standard proteins as in Fig. 61. Lane (b) 17-19dpc testis-CM. Lane (c) 17-19dpc ovary-CM. Note extra bands (*) in male CM with molecular weights between 67 and 140kDa.



M.W.'s

67kDa

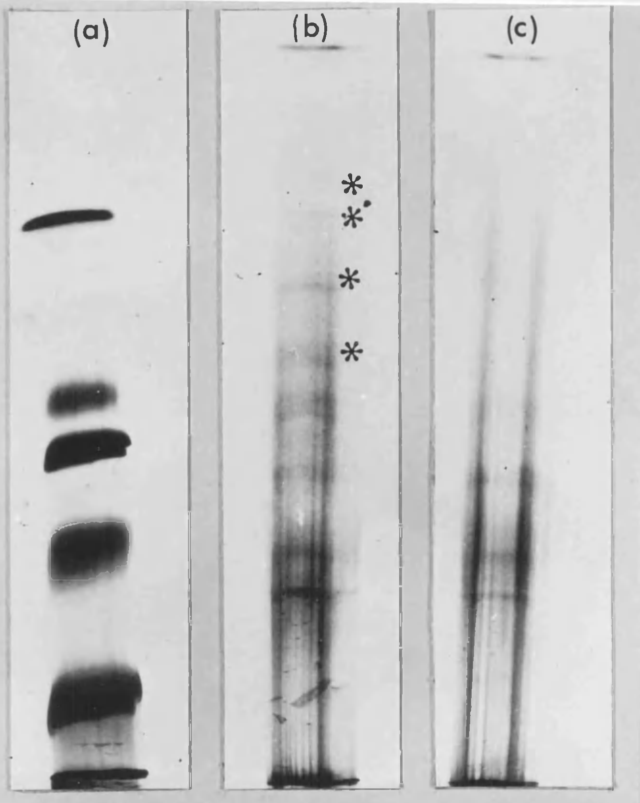
140kDa

232kDa

440kDa

669kDa

62



SECTION 6 - Phase microscope observations

This section summarises consistent observations on the progress of ovaries and testes *in vitro*.

Gonads removed and cultured from 11.5 and 12dpc settled onto the plastic substrate within 1 day of culture, ovaries to a greater extent than testes. A fibroblast-like cellular monolayer extended outwards/peripherally from settled gonads, which supported them within the culture well. Fetal tissue was more gelatinous at this stage which may have aided attachment.

It was found that, in 2 out of 3 experiments, separation of paired ovaries and testes at 11.5dpc inhibited further development. As previous studies in this laboratory have shown that indifferent gonads which are not separated develop successfully, it is concluded that the trauma to which gonads were subjected (although care was taken) was directly reponsible for failure to continue development.

Ovaries and testes cultured from 13dpc showed a difference in time of settlement when cultured for 2-4 days. 70% of ovaries settled following one day's culture, whereas equivalent testes tended to settle 24 hours later. Testes removed at 15dpc and 17dpc remained floating in the culture medium.

There was no apparent difference in time of settlement between cultures in standard and conditioned media. The 12.5-13dpc testis contained developing

testicular cords which are visible under the dissecting microscope. In addition a peripheral blood vessel also enables easy identification. These structures are even more obvious following successive days culture.

Ovaries were more fragile than equivalent testes, which develop a tunica albuginea and are consequently discrete organs at an earlier stage in development. For this reason, testes were usually subjected to a pre-incubatory treatment in a low concentration (0.01%) of EDTA, which gently dissociated tissue. Histological examination of testes revealed no inhibitory effect on cord development. Indeed in one experiment, testes (with differentiated testicular cords) which had not been pre-incubated, contained cords which were significantly more disrupted than those that had. In addition, although EDTA renders gonads more adherent to dissecting needles, attachment to the base of culture wells was not promoted.

In testes removed from 13dpc onwards, the Wolffian duct was often expanded and could be seen as a large empty oval structure, wider cranially, in the outer region of the mesonephros. This characteristic feature developed after 1-2 days culture both in testes cultured in CM and control medium. This did not appear to have a detrimental effect on cultured testes and, histologically, the cells of the duct walls appeared healthy. Presumably fluid, e.g. culture medium, built up within the duct having no exit route as the site of excision becomes sealed as the testes develop

in vitro.

As development continued, the initial elongated narrow form of the indifferent gonad altered. Testes became more rounded in appearance than equivalent ovaries, although both were more compact structures. *In vivo*, gonads project into the coelomic cavity at this stage. Testes also underwent a size increase which was not as evident in ovaries.

Testicular development was always significantly poorer than ovarian, although this was not obvious under the phase contrast microscope as peripheral tissue was always healthy. Necrotic centres, larger than those seen occasionally in ovaries, were consistently present in cultured testes. In testes excised on 14dpc, however, the extent of necrosis was reduced. It is possible that prior to this stage, successful testicular development is dependent on *in vivo* factors. In addition to the increasing size of testes, the establishment of a tunica albuginea may have inhibited diffusion of culture medium into the more central tissue (even though testes were gently dissociated with EDTA to counteract this), as peripheral tissue was always healthy. Further, testicular cord breakdown in the central necrotic areas may have led to complete cord disintegration thus spreading the necrotic effect outwards. Delayed attachment, in comparison to cultured ovaries, may also have affected development.

CHAPTER 4 : DISCUSSION

This study investigated putative factors which may be involved in the formation of both testes and ovaries. Ovary- and testis-conditioned media were generated from the culture of gonads, which released proteins into the medium.

Testis-CM (13-15dpc) was found to induce a significant reduction in germ cell number in ovaries excised on 12.5 and 13dpc. In addition a significant reduction in the total surface area occupied by testicular cords was induced by exposure of 14dpc testes to 15-17dpc ovary-CM. Adrenal-CM (14-17dpc) promoted meiosis within 13dpc ovaries, with significantly more synaptonemal complexes present than in cultured controls. The development of areas resembling a differentiating tunica albuginea beneath the surface epithelium was also noted in some ovaries removed prior to 13dpc. There was no evidence for a Meiosis Preventing Substance within the ages of CM generated. The differentiation of Sertoli, Leydig and peritubular myoid cells was not noted. Although AMH and testosterone are believed to be under production within testes used to generate conditioned media, female genital ducts developed normally.

Neither ovary-CM nor adrenal-CM induced precocious entry into meiotic prophase, even though testicular cords were frequently disorganised or completely disrupted. Culture of testes in CM generated from older testes had no beneficial effect on cord development. As in ovaries, ducts

developed normally.

Isolated germ cells of both sexes were successfully cultured within conditioned media generated from the same and opposite sex. Viable germ cells were maintained for up to 7 days.

Electrophoretic analysis of conditioned media revealed distinct differences between ovary- and testis-CM, with bands of a higher molecular weight in males than in equivalent females.

Results suggested the involvement of a diffusible substance in tunica albuginea induction in fetal ovaries. A tunica albuginea was identified if 3-4 layers of flattened cells and connective tissue were evident beneath a flattened surface epithelium, anywhere along the outer aspects of cultured ovaries. This definition was strictly adhered to at all times. Within all 11.5dpc ovaries cultured for 7 days in 17-19dpc testis-CM, signs of tunica albuginea development were evident. Further, no areas of tunica albuginea-like formation were noted in ovaries cultured in 13-15dpc testis-CM. Testis-CM generated at different stages (14-15dpc, 15-17dpc) also induced tunica albuginea development in cultured ovaries (12.5dpc, 12dpc). However, the effect was not evident in all experimental ovaries. When the experiments were established, it was not known which conditions (i.e. variance in age of CM used, age and number of explants and duration of culture) would provide an effect. These results cannot, therefore, be

analysed statistically. However, the induction of tunica albuginea-like formation in all 11.5dpc ovaries cultured for 7 days suggests that either the early stage of differentiation or the prolonged culture period may be involved. The absence of a similar effect in ovaries removed at 13dpc and cultured for 4 days may be due to either the later stage of differentiation or the shorter culture period. Only one 13dpc ovary was examined after 7 days with no sign of tunica induction, although this is obviously inconclusive. There was no sign of tunica albuginea formation in any ovaries explanted on 13dpc in any of the culture conditions. It would be interesting to culture 11.5dpc ovaries in all ages of testis-CM for varying periods up to 7 days in order to determine whether sexually indifferent ovaries are more susceptible to the factor involved. Further experiments involving different ages of ovaries cultured for 7 days would determine whether length of culture was important in tunica albuginea induction.

Merchant (1975) stated that an early and notable event in the development of the testis was the separation of the developing cords from the surface epithelium by the formation of a tunica albuginea. The development of a tunica in 12.5dpc ovaries co-grafted with equivalent testes under the kidney capsule of adult mice was also noted by Burgoyne *et al.* (1986) after 6 days. However, this may have been an extension of the developing tunica around the testis.

Taketo *et al.* (1985a) did not report tunica development in 12dpc ovaries grafted singly beneath the kidney capsule of adult males. Vigier *et al.* (1987) found that a layer of flattened connective cells resembling a differentiating tunica albuginea developed in ovaries cultured in AMH at concentrations of 1.5μg/ml-1 or more. Benhaim *et al* (1982) found that fetal ovaries from postnatal calves developed a tunica albuginea-like structure when cultured in male lymphoma (known to contain H-Y antigen) and teratoma supernatant.

An additional effect of 14-15dpc testis-CM noted in 12.5dpc ovaries cultured for 4 days was a reduction in germ cell number, although this would require statistical verification by further quantitative analysis. Ovarian differentiation had proceeded with meiotic oocytes present, equivalent to those within a 16-17dpc ovary, but ovigerous cord formation was not as advanced as might be expected.

More significant results were obtained with 13dpc ovaries cultured in 13-15dpc testis-CM. Following a 4 day culture period, ovaries contained approximately half the number of oocytes present in contralateral ovaries cultured in control medium. The diffusible factor was removed following heat-inactivation, suggesting that it may be a protein, but not by filtration with 0.22μm Millipores.

In the present study, somatic cells were mainly healthy and occasional mitoses indicated the vitality of tissue. In addition, pyknotic cells were not more numerous

in experimentals than in cultured controls, suggesting that germ cell death was not a major contributory factor and that the inhibitory effect was specifically on germ cell mitosis. However, the possibility that degenerated oocytes had been resorbed prior to fixation cannot be ruled out. In addition mitotic indices were not determined and this should be carried out in future studies. Vigier et al. (1987) counted numbers of germ cells within fetal ovaries at various stages during the culture period in AMH-supplemented medium. As in the current study, a statistically significant reduction in germ cell number was noted. In addition, it was found that following entry into meiosis, the rate of degeneration was similar in controls and AMH-treated ovaries.

That ovarian development continues in the absence of large numbers of germ cells has been shown both by germ cell cytotoxic studies and through examination of genetic mutants, although follicles fail to form in the absence of oocytes (Merchant, 1975; Tam and Snow, 1981; Mintz and Russell, 1957; McCoshen and McCallion, 1975, Taketo et al., 1985a). In the present study ovarian differentiation was achieved in 13dpc ovaries cultured in 13-15dpc testis-CM, with the smaller ovigerous cords resembling those of a 16-17dpc ovary. Moreover, organisation proceeded, even when the full complement of oocytes was absent in experimental explants. In these ovaries there was a slight but non-significant reduction in gonadal volume. Addition of purifed bovine AMH to culture medium produced a

statistically significant reduction in rat gonadal volume (Vigier _et al._ 1987; 1988). An additional effect of culture both in rat testis-CM and in control media was a slight reduction in gonadal volume (Prépin _et al._, 1985a; Prépin and Hida, 1989a). This was also observed in transplanted ovaries where oocyte loss was accompanied by a marked reduction in gonadal volume (Ozdzenski _et al._, 1976; Burgoyne _et al._, 1986).

Current results also showed that germ cell number was not reduced in 11.5dpc ovaries cultured for 7 days in 17-19dpc testis-CM, or in 12dpc ovaries cultured in 13-15dpc or 15-17dpc testis-CM. In addition, 13dpc ovaries cultured in 17-19dpc testis-CM showed no apparent reduction in germ cell number after two days culture. The inhibitory effect may require a longer period before it becomes evident.

Further experiments varying the duration of culture would determine the time when the inhibitory effect is most pronounced. Prépin _et al._ (1985b) found that the reduction effect was most obvious between the 3rd and 4th day _in vitro_ when 13.5dpc rat ovaries were co-cultured with testes or in testis-CM. This was equivalent to the final period of mitotic replication prior to entry into meiosis, and is in agreement with the hypothesis that the inhibitory effect is one specifically on mitosis and not meiosis.

Previous studies, using the rat as an experimental subject, have shown that 13.5dpc fetal ovaries, cultured

in medium conditioned by the culture of 16.5dpc and postnatal testes for 4 days, contained reduced numbers of oocytes (Prépin _et al._, 1985a; Prépin and Hida, 1989a). Conditioned medium was sterilised by two successive filtrations (0.8μm then 0.22μm) (Prépin _et al._, 1985a). This agrees with the present study, with no removal of the inhibitory factor following filtration and a reduction in the number of oocytes and small ovigerous cords within the cultured ovaries.

Prépin's group used testis-CM which was generated from rat testes of 16-20dpc to 4-64 days post partum (dpp). No difference in the germinostatic effect was noted. This suggested that the factor involved may have a major role in normal testicular development. The meiotic state of oocytes was not mentioned. Taketo _et al._ (1985a) also found reduced numbers of germ cells within 12.5dpc ovaries transplanted under the kidney capsule of adult male mice, suggesting that the effect is a general male effect rather than specifically a testicular one. Large macromolecules or direct cell-cell interactions were thought to be involved (Taketo-Hosotani, 1987). However, Taketo-Hosotani and Sinclair-Thompson (1987) also found that ovaries transplanted into adult female mice developed similarly, although at a lower incidence than within males. It was suggested that the mesonephros had a protecting influence from the 'masculinising' effect of female hosts. Diffusible proteins (≥ 50KDa) were also implicated as being responsible for a reduction in germ cell number within

fetal and postnatal rat testis-CM (Prépin *et al.*, 1985a).

In the rat, a reduction in the female germ cell population was induced by co-cultured testes explanted on 16.5dpc but not 13.5dpc (Charpentier and Magre, 1989). Prépin and Hida (1989b) found that 9 day old ovaries, co-cultured with 13.5dpc ovaries also had an inhibitory effect on oocyte number.

Vigier *et al.* (1987) examined the effect of adding AMH to cultures of rat fetal ovaries. A threshold concentration of approximately 1μg/ml-1 was sufficient to decrease germ cell number, with an 82% reduction at concentrations of 3μg/ml-1. Signs of Müllerian duct regression also occurred at around 1μg/ml-1, with complete regression at the level of the gonads at 1.5μg/ml-1. Recently, Behringer *et al.*, (1990) have produced transgenic mice expressing human AMH. These authors concluded that a higher level of AMH is apparently needed to produce the ovarian effect (including a decrease in germ cell number) than is required for the Müllerian duct effect, since ovarian development can occur when AMH levels are sufficient to inhibit Müllerian duct differentiation. Charpentier and Magre (1989) investigated the effects of co-cultured testes on ovaries *in vitro*. They found that at 13.5dpc (when little AMH is produced) only Müllerian ducts regressed, whereas later testes (16.5dpc) which produced large amounts of AMH induced both duct regression and a reduction in germ cell number. However, only a loss in germ

cells was observed when 16.5dpc testes were cultured at a distance from the ovaries. The authors concluded that a testicular factor distinct from AMH was responsible for the observed effect on germ cell number. Prépin and Hida (1989a) reported that 14.5dpc rat ovaries, cultured in testis-CM and containing reduced numbers of oocytes, developed AMH activity following 16 days in culture, but did not develop a germinostatic effect.

This thesis showed that oocyte number was decreased without Müllerian duct inhibition. This indicates that it is unlikely that AMH is responsible for producing these two effects at different concentrations, since one would expect Müllerian duct regression at concentrations producing a reduction in germ cell number. The effects of AMH may be modified, however, by the presence of other diffusible male and/or growth factors present in the CM.

The results show that mouse testis CM produced a similar germinostatic effect to that noted in the rat, although, unlike the rat, this was not a universal feature of the different stages of conditioned media generated in this study. The factor(s) involved remains to be elucidated.

Results also show that within 13dpc ovaries cultured for 7 days in 13-15dpc testis-CM, oocytes were isolated within developing follicles, even though oocyte number was significantly reduced after 4 days, and Sertoli cell differentiation was not noted.

In the present study, conditioned media, generated

from twice as many male mouse fetal adrenal glands as female, showed no reduction in germ cell number when used to culture 13dpc ovaries for 4 days. Further controls could use other male organ-conditioned media to confirm or disprove the non-specific male effect. In addition, as adrenal glands were cultured for the same length of time (2 and 3 days) as testis-CM, it is unlikely that depletion of nutrients in the culture medium was responsible for the inhibitory effect.

The differentiation of cells resembling fetal Sertoli cells in the interstitial tissue of cultured fetal ovaries, and their aggregation into testicular-cord like structures, has been noted only in experiments where 16.5dpc rat testes were cultured in close contact with 13.5dpc ovaries (Charpentier and Magre, 1989). It is interesting to note that testicular-like cords also developed in rat ovaries that had never been subjected to a 'masculinising' influence. 14.5dpc rat ovaries cultured for a prolonged period in testis-conditioned medium (both fetal and postnatal) and control medium developed cords more characteristic of equivalent testes (Prépin and Hida, 1989a).

Prépin and Hida (1989a) found that testicular cords did not develop in ovaries cultured in testis-CM for only 4 days, but appeared after 12 days. It would be interesting to investigate the development of testicular cord-like structures in the mouse by culturing ovaries for an

equivalent period.

It has been suggested that epithelial cords develop from follicle cells that become diverted from their normal development as a direct result of germ cell depletion (Ozdenski et al., 1976; Prépin and Hida, 1989a; Benhaim et al., 1982; Taketo et al., 1985a). Hashimoto et al (1990) recently found that female somatic cells isolated from fetal mouse ovaries differentiated into testicular cord-like structures in the absence of germ cells. Taketo et al. (1985a) found that all the cell types characteristic of a testis differentiated within indifferent ovaries transplanted beneath the kidney capsule of adult males. The best developed seminiferous cords contained no oocytes. It was suggested that oocytes degenerated as a direct consequence of Sertoli cell differentiation. However, McLaren (1980) detected growing oocytes within the tubules of sex-reversed male mice, which would also be composed of XX Sertoli cells. Müller and Schindler (1983) suggested that a cascade of events is initiated by the primary differentiation of the pre-Sertoli cells, which are themselves induced by a diffusible testis-specific factor.

Undoubtedly, investigation at the level of gene mapping has given great insight into the determination of sex. The gene products which may be involved, both in the overall determination of sex and formation of sex-specific morphology remain to be elucidated. McLaren (1987a) suggested that pre-Sertoli cell differentiation is dependent not only on Tdy, as in one XX<->XY female

chimaeric mouse, XY cells also contributed to the follicle cell population.

In 13dpc ovaries cultured for 4 and 7 days in 13-15dpc testis-CM, somatic cells surrounding oocytes appeared mainly healthy with no sign of pre-Sertoli cell differentiation, even though oocyte number was reduced. Many oocytes within the latter cultures were, in fact, encompassed within developing unilaminar follicles.

In no experiment was duct regression noted that was not characteristic of the sex of the gonad. Expanded mesonephric ducts were occasionally noted in cultured testes. These have also been found by others and are presumably a consequence of poor fluid drainage (Taketo et al., 1984a; Burgoyne et al., 1986). In 12dpc gonads cultured for 2 days the Müllerian duct was not always obvious. Dyche (1979) has stated that both pairs of ducts are present at 12dpc. However, staging of fetuses differed slightly, being approximately 24 hours earlier with the method used in present study. Regression of the appropriate ducts, in both males and females, commences at around 13dpc in vivo (according to Dyche, 1979). It would, therefore, be expected that there would be little sign of regression within cultured ovaries. Although Leydig cell differentiation occurs within the 13-15dpc mouse testis, female Wolffian duct maintenance was never noted. It is probable that insufficient quantities of testosterone were present in the CM. AMH synthesis begins soon after,

testicular cord differentiation (Jost and Magre, 1984), female Müllerian ducts, however, developed normally, with no sign of regression. Others have produced similar results. Müllerian duct regression has never been induced by CM; only when testes were cultured in close contact with ovaries, or their ducts in Picon's bioassay (Picon, 1969), was regression induced (Charpentier and Magre, 1989). This suggests that the effect may be cell-mediatied, or that AMH may be modified by other substances present within CM.

The normal process of meiosis was not affected by CM generated from fetal testes or ovaries. Vigier et al. (1987; 1988) found that AMH no effect on the progression of meiosis in fetal rat ovaries.

Although FCS was added to cultured adrenals rather than to the conditioned medium as in gonadal conditioned media, depletion of FCS components in adrenal CM was not noted as ovarian development was not adversely affected, although testicular development was poorer.

Meiotic male germ cells were not noted in any testis cultures, either within adrenal-CM or ovary-CM. The meiosis-inducing effect observed by Zamboni and Upadhyay (1983) may be mediated by cell-cell contact or, as the authors suggested, in the absence of a testicular environment. As germ cells were enclosed within a testicular environment and, at least initially, within cords, this alone could explain the observations, although male germ cells may have been committed to develop as T-prospermatogonia at the times of excision. If a MIS is

produced by the fetal adrenal gland, this would have to be concentrated enough to overcome the inherent meiosis preventing action within the testis.

It has been suggested that all germ cells that fail to become enclosed within the testicular cords enter meiosis (Byskov, 1978b) although McLaren (1984) disputes this. In addition, others have suggested that disruption of cords allows male germ cells to begin meiosis (Ozdzenski and Presz, 1976), although Stein and Anderson (1981) found that few viable germ cells remained in bisected testes, and precocious meiosis was not oberved. Grinsted et al. (1979) found that a 4 day culture period was sufficient for induction of meiosis in fetal mouse testes cultured in conditioned medium generated from pubertal bull testis, rete testis and epididymis. However, approximately half contained disrupted cords. It was suggested that MIS was responsible. Meiosis was also induced within mouse testicular cords when testes were co-cultured transfilter to pubertal mouse tissue. Again, in controls, meiotic germ cells were noted outwith the testicular cords.

In the present study, even though germ cells were frequently located outwith discrete cords, meiosis was not induced. This is in agreement with Taketo et al. (1984a) who reported that testicular cord development was prevented by addition of cAMP analogues to cultures of 12dpc mouse testes did not result in early entry into meiotic prophase.

Meiosis was not inhibited in ovaries cultured in

testis-CM. Byskov and Saxén (1976), found that meiosis was inhibited in 14dpc mouse ovaries co-cultured with 14dpc testes for a period of 7 days, with many pyknotic oocytes present within the tissue. These authors concluded that MPS acted to inhibit the oocytes which had already entered meiosis.

Mitotic divisions of the female germ cells occur for a period of 3 or more days, preceding entry into meiotic prophase (McLaren, 1984). In the strain of mouse used in this study, 14.5dpc was considered to be the time when female mouse germ cells become committed to meiosis, which agrees with the time proposed by Taketo _et el._ (1984b) and Godin _et al._, (1990). Synaptonemal complexes have been reported at 17 dpc (Baker and Neal, 1973; Evans _et al._, 1982; Upadhyay and Zamboni, 1982). Current results showed that oocytes continued through meiotic prophase, reaching the zygotene/pachytene stage in 11.5dpc ovaries cultured for 7 days; 12.5dpc ovaries cultured 4 days and 13dpc ovaries cultured for 4 days in varying ages of testis-CM. Synaptonemal complexes were evident within oocyte nuclei of both experimental and control cultures. This is in agreement with Taketo and Koide (1981) who also found that germ cells within mouse ovaries removed on the 11th day of gestation and cultured for 7 days reached the zygotene/pachytene stage of meiosis.

Ovarian development was markedly better than equivalent testes within adrenal-CM. The number of synaptonemal complexes was significantly increased in 13dpc

ovaries cultured for 4 days in 14-17dpc adrenal-CM. In addition, 13dpc ovaries appeared advanced in development after 2 days culture in 14-16dpc adrenal- CM although this 'advantage' was lost after day 4. These results confirm and extend the findings of Upadhyay and Zamboni (1982), revealing the beneficial effects of an adrenal environment on germ cell development along the female pathway.

In order to investigate the entry of male germ cells into meiotic prophase it would be interesting to culture isolated male germ cells in varying concentrations of adrenal CM and standard culture medium. De Felici and McLaren (1983) found that female germ cells progressed through meiotic prophase when isolated at the onset of meiosis (13.5dpc). Equivalent male germ cells did not enter meiosis in vitro, suggesting either that culture conditions were not optimum, or that meiosis must be induced, perhaps at an earlier stage in development. Both male and female germ cells failed to enter meiosis when isolated at the sexually indifferent stage, but mitotic divisions continued. However, neither did meiosis progress in older female germ cells due to the low temperature the authors found necessary for prolonged survival in vitro. In the present study, morphological analysis of cultured isolated germ cells was attempted in order to investigate effects on meiosis. However, following processing and embedding in resin, it was found that germ cells no longer remained attached to the fibronectin-coated coverslip.

The presence of residual gonadal tissue *in vitro* has been shown to have a beneficial effect on the survival of primordial germ cells (PGCs) of the same sex (Tavendale *et al.*, 1987). Godin *et al.* (1990) have found that CM prepared from undifferentiated (10.5dpc) and unsexed genital ridges, increased the number of PGCs in culture, but did not extend the survival period, with no germ cell survival after 7 days. No serum was added to the conditioned medium. Interestingly, the method of CM-generation was similar to the one employed in the present study, although their CM was probably less concentrated. It would seem that there is a diffusible substance present within genital ridges which is beneficial for isolated germ cell mitosis prior to sexual differentiation. Taketo *et al.* (1986) reported that, in the 11dpc mouse, serum components were required for preservation of male germ cells within developing testicular cords *in vitro*, but not their proliferation. In the absence of serum, 11dpc testes contained reduced numbers of germ cells (Taketo *et al.*, 1986). These results suggest that serum contains some component(s) found within *in vivo* environment that are beneficial for germ cell differentiation. Addition of serum to conditioned media should both increase the number of isolated germ cells and preserve them for a longer period.

This study showed that both male and female germ cells isolated between 11.5 and 13dpc were successfully cultured in 14-15dpc testis- and ovary-CM, respectively, (with added fetal calf serum) with viable germ cells

remaining after 7 days, although overall numbers were reduced. The isolation and maintenance of germ cells theoretically allows investigation of putative control factors present within the in vivo environment. However, the successful maintenance of germ cells for long periods has proved difficult (De Felici and McLaren, 1982; 1983). Analysis of CM may give insight into the factors involved in maintaining germ cell viability. Purification and concentration of these hypothetical substances may aid further investigation into environmental control of germ cell differentiation.

Morphologically, there were no apparent signs of masculinisation, other than signs of tunica formation, in 12dpc ovaries cultured in 13-15dpc and 15-17dpc testis-CM for 2 days. Ovaries were well-maintained and showed signs of organisation, indicating that development had proceeded. This is earlier than reported by Upadhyay et al. (1979) who found that connective tissue septa invaded ovaries from 16dpc. However, the authors do not describe ovarian morphology between 14 and 16dpc. Taketo et al. (1984b) found that ovaries removed on the 12th day gestation more closely resembled the equivalent in vivo situation than those removed a day earlier.

A frequent finding within 2 day cultures (12dpc and 13dpc cultured ovaries) was the presence of greatly expanded blood vessels around groups of germ cells. They may be a sign of masculinisation as larger blood vessels

develop within the testis as compared to the ovary. Another possible explanation is that they are an immediate reaction to the trauma of excision. Further, in those testes removed on 12dpc and cultured in 15-18dpc ovary-CM, blood vessels were not as expanded as in ovaries cultured in testis-CM.

In the present study development continued within 11.5dpc ovaries cultured in both control medium and 17-19dpc testis-CM for 7 days. Ovaries resembled those within 17dpc fetuses, although a tunica developed in all 3. Germ cells were not inhibited and nuclei contained synaptonemal complexes characteristic of the 17dpc ovary. Taketo and Koide (1981) also noted that ovaries cultured from the 11th day of gestation for 7 days contained oocytes which had progresses to the pachytene stage. However, variable results were produced with cultures initiated at 11.5dpc. In one experiment, differentiation did not proceed and it was concluded that this was due to trauma encountered on separation of the paired complexes which are very fragile at this stage. This was in order to provide an accurate culture control for comparison.

Structural development in ovaries was retarded by approximately 0.5 to 1.5 days in cultures maintained for longer than 2 days, as was stated by Taketo et al. (1985b) and confirmed by Mackay and Smith (1989). However, in the rat, Prépin and Hida (1989a) found no such retardation in 14.5dpc ovaries cultured for 7 days.

Upon sexual differentiation of the testes, germ cells

become enclosed by pre-Sertoli cells in the testicular cords (approximately 12.5dpc). Complex contact zones form soon after which effectively isolate the spermatogonia from the surrounding tissue. When testes containing testicular cords were removed from the in vivo environment on 12, 13 and 13.5dpc, their integrity was affected, although to a lesser extent in control cultures than experimentals after 4 days. However, testicular development was markedly improved when removed at 14dpc, i.e. possibly after the time of testicular cord stabilisation. Further, in 14dpc testes cultured in 15-17dpc ovary-CM a significant reduction in the total surface area occupied by testicular cords was apparent. This reduction did not appear to be confined to the germ cell population as no spaces devoid of germ cells were present within cords. It can be concluded, therefore, that removal and culture of testes excised before 14dpc, for periods longer than 2 days leads to cord breakdown in both control medium and ovary-conditioned media in the conditions used in this study.

Testicular cords were not prevented from forming in control testes removed at the time of incipient testicular cord formation (i.e. 12dpc) and cultured for 2 days, but they were disorganised. Cords formed in 2 12dpc testes cultured together with an ovary for 2 days within 15-18dpc ovary-CM. However, ovary-CM (15-18dpc) apparently inhibited cord formation in one 12dpc testis cultured alone. Interestingly, 12dpc testes cultured in 15-18dpc

testis-CM did not show any developmental advancement and resembled the testis cultured alone in 15-18dpc ovary-CM. The apparent inhibition of cord formation was not a sex-specific effect. In addition, a 12dpc ovary cultured within 15-18dpc ovary-CM resembled equivalent ovaries cultured in 15-17dpc testis-CM. Unlike testes, development seemed unaffected by CM generated from either sex. This may reflect the susceptibility of indifferent testes to culture.

Mackay and Smith (1986) successfully cultured 11.5dpc testes for 7 days, although paired urogenital complexes were not separated. However, Burgoyne et al. (1986) cultured 11.5dpc testes transfilter to both testes and ovaries of an equivalent age and found that testicular development was poor with disorganised testicular cords. Others have found that testes removed at 12dpc survived and developed well-organised testicular cords following 7 days culture (Taketo et al., 1984a).

When 11dpc gonads are cultured for 2 days in media that is serum-free, then subsequently serum added, testis cords form but contain few or no germ cells indicating that serum is essential for the preservation of germ cells (Taketo et al., 1986). Others have shown that serum is not a prerequisite of testicular cord formation, although serum-containing medium did not prevent cords forming in the mouse (Mackay and Smith, 1989) as has been reported in the rat (Agelopoulou et al., 1984). In the current study, FCS was added to testis cultures from 12dpc to

14dpc.

In the rat, it has been reported that addition of FCS to culture medium delayed and disrupted developing testicular cords (Agelopoulou et al., 1984; Magre and Jost; 1984). However, in the present study, no testicular cord disruption was noted in cultures initiated at 14dpc when cords are already present. Neither was cord formation prevented (at least initial stages), as in earlier testes, cord-like structures were often noted. Stein and Anderson (1981) also cultured rat testes (excised prior to testicular cord formation) in FCS-supplemented culture medium and found that testicular differentiation proceeded in over 50% of cultures.

In the present study general development was poorer in testes removed before 14dpc than in ovaries, in both control and experimental cultures. This may be due to the comparatively large volume of testes. Further, germ cells are enclosed within distinct testicular cords and it is possible that any disruption of these by developing necrosis in the depths of the tissue could lead to complete cord breakdown, as was seen in all cultures maintained longer than 2 days.

In vitro experiments on fetal gonadal differentiation have proved invaluable for examination of the normal processes involved *in vivo* (Picon, 1969; Taketo and Koide, 1981; Vigier et al. 1987). Evans et al. (1982), however, found that testicular cord diameter was reduced, in

cultured mouse testes, as was the number of pre-Sertoli cells. Baker and Neal (1973), in the mouse, found that numbers of germ cells were reduced if ovaries were cultured between 14 and 18dpc, indicating the need for gonadal components absent from culture medium.

This problem has been overcome to a certain extent by the use of transplantation techniques (Turner and Asakawa, 1964; Ozdzenski _et al._, 1976; Ozdzenski and Presz, 1984; Burgoyne _et al._, 1986). However, this also has disadvantages in that continual monitoring of grafted gonads is impossible and there are also problems in recovery at the end of the, usually extensive, period _in vivo_. For example, out of 163 transplanted heterosexual gonadal pairs, only 5 grafts were recovered with both ovary and testis present (Ozdzenski _et al._, 1976).

The present study used _in vitro_ techniques to culture gonads for both short periods (2 days) and more prolonged periods (4 and 7 days). The 2 day culture period was found to be insufficient for gonads to develop far following the trauma of explantation. However, useful results were obtained (tunica albuginea-like formation in ovaries; testicular cord formation in testes). A longer period of culture, also produced interesting results, particularly with regard to ovarian development (tunica development; entry into meiotic prophase; beginning of follicle development). For the purposes of the present study, 4 days was found to be sufficient to produce specific CM effects.

Electrophoretic analysis of CM revealed consistent bands in 13-15dpc testis-CM and also in one sample of 13-15dpc ovary-CM. These proteins may have a general role in gonadal development. Bands exclusive to 13-15dpc testis-CM were also noted which were of a higher molecular weight than those unique to equivalent ovary-CM. Additional proteins appeared in ovary-CM cultured for two more days which may reflect developmental changes between 15 and 17dpc.

Conditioned media produced from older stages of testes (17-19dpc) contained proteins of a lower molecular weight than within ovary-CM. A band in the region of that expected for AMH was apparent. These results clearly warrant further investigation. The molecular weight of AMH is approximately 140kDa. No bands of this molecular weight were noted in 13-15dpc testis-CM. However, it is possible that only a low concentration of AMH was present.

Cole et al. (1982) used 2-D gel electrophoresis to examine germ cell proteins at the beginning of oogenesis. Three stage-specific changes in proteins were detected at 12, 14 and 17dpc. One protein present in 12dpc female germ cells and gonadal somatic cells disappeared by day 14. Two proteins appeared in 14dpc female germ cells but not somatic cells. No stage-specific changes in proteins were noted, however, in preparations of male germ cells of the same age.

In summary, the present results show that mouse

testis CM contains proteins absent from ovary-CM and produces a germinostatic effect on female germ cells. This effect is similar to that noted in the rat, with the number of oocytes significantly reduced in experimental cultures, although this did not affect ovarian organisation. Taketo-Hosotani (1987) suggested that macromolecules may be implicated in reducing germ cell number in transplanted fetal mouse ovaries. Prépin et al. (1985a) showed that the reduction effect of fetal and postnatal rat testis-CM was retained following dialysis with a membrane cut-off of 1-50,000 daltons. The lowest range of PhastGel Gradient 8-25 was 50,000, therefore the proposed substance could be present.

Vigier et al. (1987) found tunica development in ovaries exposed to AMH at concentrations where Müllerian ducts were completely regressed and oocyte numbers and gonadal volume reduced. In the present study, 12.5dpc ovaries, which appeared to contain reduced numbers of germ cells also developed areas of tunica albuginea development. However, 13dpc ovaries, in which the germ cell loss was confirmed quantitatively, did not develop tunica-like regions and gonadal volume was not significantly reduced. Müllerian duct regression was also not induced. These results suggest that AMH is not the diffusible factor involved in either tunica albuginea induction or germ cell number reduction in fetal ovaries in this case.

In the present study, gonadal volume was not significantly reduced in experimental ovaries; this result

differs from that in the rat, where others have noted a slight decrease in gonadal volume (Prépin _et al._, 1985a; Vigier _et al._, 1988).

It has been noted that there is a change in the cell surface components of PGCs at the time of sexual differentiation (Heath and Wylie, 1981; Eddy and Hahnel, 1983). Hashimoto _et al._ (1990) suggested that female somatic cells have the capacity to differentiate into testis cords on 12.5dpc but lose it after 13.5dpc. In the present study, the somatic component of both ovaries and testes apparently changed in some way after a certain stage in development. In 13dpc ovaries, the surface epithelium seemed less susceptible to masculinisation than those removed between 11.5 and 12.5dpc. Testicular cords remained intact within cultured testes removed at 14dpc, but not before.

PGCs are at all times closely associated with somatic cells (De Felici and McLaren, 1983). A substrate allowing attachment was provided for cultures of isolated germ cells in the form of fibronectin, shown to be present along the migratory route of migrating germ cells (Merchant-Larios and Alvarez-Buylla, 1986). Recently, a reconstituted basement membrane gel was used to culture and follow the development of Sertoli cells (Hadley _et al._, 1990). This could also be used in the culture of germ cells.

This thesis investigated the involvement of factors generated by ovaries and testes on the development of

gonads of the opposite sex; it would also be interesting to further extend the investigation into effects of varying ages of CM on gonads of the same sex. In addition, in order to investigate further the induction of meiosis, the effect of CM produced from gonads explanted between 10 and 12dpc (i.e. before entry into meiosis in the ovary and possibly committal to either male or female germ cell development) could be investigated. Further, the effects of CM from adrenal glands, ovaries and testes on gonads removed between 10 and 12dpc should also be studied. In future studies, the effects shown to be produced by different conditioned media could be further investigated by both feeding cultures and alternating the sex and age of CM during the culture period.

In addressing the original aims detailed in the opening chapter, the following conclusions may be drawn. Ovary-CM induced a reduction in the total surface area occupied by testicular cords within 14dpc testes after 4 days. The presence of diffusible factors promoting meiosis was confirmed in ovaries cultured in adrenal-CM. This supports the suggestion that the adrenal gland provides a favourable environment for the promotion of germ cell development along the female pathway (Upadhyay and Zamboni, 1982). Meiosis was not, however, induced in testes cultured in either adrenal- or ovary-CM, even when germ cells were not enclosed within testicular cords.

There was no inhibition of meiosis evident within ovaries cultured in testis-CM. This suggests that meiosis

inhibition may be mediated by cell-cell associations.

Finally, diffusible factors were shown to be present within testis-CM which affected the development of cultured ovaries. Tunica albuginea formation was noted in a number of ovaries cultured in testis-CM; the effect may depend on either the age of the ovary or the duration of culture. A significant reduction in germ cell number was shown to be induced following exposure of 13dpc ovaries to 13-15dpc testis-CM for 4 days. Male germ cells enter mitotic arrest between 13 and 15 dpc; interestingly, this corresponds to the time of generation of the testis-CM: any inhibitory factors controlling mitosis might be expected to be released during this period.

CHAPTER 5 - REFERENCES

Agelopoulou, R., Magre, S., Patsavoudi, E. and Jost, A. (1984) Initial phases of the rat testis differentiation in vitro. Journal of Embryology and experimental Morphology, **83**, 15-31.

Baker, T.G. & Neal, P. (1973) Initiation and control of meiosis and follicular growth in ovaries of the mouse. Ann. Biol. anim. Bioch. Biophys., **13**, 137-144.

Barka, T. & Anderson, P.J. (1962) In Histochemistry, Theory, Practice & Bibliography, p187, New York: Hoeber.

Beaumont, H.M. & Mandl, A.M. (1962) A quantitative and cytological study of oogonia and oocytes in the foetal and neonatal rat. Proceedings of the Royal Society of London. Series B., **155**, 557-579.

Behringer, R.R., Cate, R.L., Froelick, G.J., Palmiter, R.D. & Brinster, R.L. (1990) Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. Nature, **345**, 167-170.

Benhaim, A., Gangnerau, M.N. Bettane-Casanova, M., Fellous, M. & Picon, R. (1982) Effects of H-Y antigen on morphologic and endocrine differentiation of gonads in mammals. Differentiation, **22**, 53-58.

Bhuyan, B.K., Loughman, B.E., Fraser, T.J. & Day, K.J. (1976) Comparison of different methods of determining cell

viability after exposure to cytotoxic compounds. Experimental Cell Research, **97**, 275-280.

Blandau, R.J., White, B.J. & Rumery, R.E. (1963) Observations on the movements of the living primordial germ cells in the mouse. Fertility and Sterility, **14**, 482-489.

Burgoyne, P.S., Ansell, J.D. & Tourney, A. (1986) Can the indifferent mammalian XX gonad be sex-reversed by interaction with testicular tissue? In Development and Function of Reproductive Organs, eds. Eshkol, A., Dekel, N., Eckstein, B., Peters, H. & Tsafriri, A., pp23-39, Serono Symposia Review No. 11. Ares-Serono Symposia.

Burgoyne, P.S., Buehr, M., Koopman, P., Rossant, J. & McLaren, A. (1988) Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX <-> XY chimaeric mouse testes. Development, **102**, 443-450.

Byskov, A.G. & Saxén, L. (1976) Induction of meiosis in fetal mouse testis in vitro. Developmental Biology, **52**, 193-200.

Byskov, A.G. (1978a) The anatomy and ultrastructure of the rete system in the fetal mouse ovary. Biology of Reproduction, **19**, 720-735.

Byskov, A.G. (1978b) The meiosis-inducing interaction between germ cells and rete cells in the fetal mouse gonad. Ann. Biol. anim. Bioch Biophys, **18**, 327-334.

Challoner, S. (1975) Studies of oogenesis and follicular development in the golden hamster. 2. Initiation and control of meiosis in vitro. Journal of Anatomy, **119**, 149-156.

Charpentier, G. & Magre, S. (1989) La réduction du nombre de cellules germinales induite in vitro par le testicule dans l'ovaire foetal de rat pourrati etre due a un facteur distinct de l'hormone anti-Müllérienne. Comptes Rendus Académie des Sciences. Paris, Série III, **309**, 35-42.

Chartrain, I., Magre, S., Maingourd, M. & Jost, A. (1984) Effect of serum on organogenesis of the rat testis in vitro. In Vitro, **20**, 912-922.

Chiquoine, A.D. (1954) The identification, origin and migration of the PGCs in the mouse embryo. The Anatomical Record, **118**, 135-146.

Clark, J.M. & Eddy, E.M. (1975) Fine structural observations on the origin and associations of primordial germ cells of the mouse. Developmental Biology, **31**, 275-284.

Cole, R.E., Canfield, T.K. & Gartler, S.M. (1982) Patterns of protein synthesis at the onset of oogenesis in the mouse. Differentiation, **22**, 85-89.

De Felici, M. & McLaren, A. (1982) Isolation of mouse primordial germ cells. Experimental Cell Research, **142**, 476-482.

De Felici, M. & McLaren, A. (1983) In vitro culture of mouse primordial germ cells. Experimental Cell Research, **144**, 417-427.

De Felici, M., Boitani, C. & Cossu, G. (1985) Synthesis of glycoconjugates in mouse PGCs. Developmental Biology, **109**, 375-380.

Diwan, S.B. & Stevens, L.C. (1976) Development of teratomas from the ectoderm of mouse egg cylinders. Journal of the National Cancer Institute, **57**, 937-939.

Donahoe, P.K., Ito, Y. & Hendren III, W.H. (1977) A graded organ culture assay for the detection of Müllerian inhibiting substance. Journal of Surgical Research, **23**, 141-148.

Donovan, P.J., Stott, D., Cairns, L.A., Heasman, J. & Wylie, C.C. (1986) Migratory and postmigratory mouse primordial germ cells behave differently in culture. Cell, **44**, 831-838.

Dyche, W.J. (1979) A comparative study of the differentiation and involution of the Müllerian duct and Wolffian duct in the male and female fetal mouse. Journal of Morphology, **162**, 175-210.

Eddy, E.M. & Hahnel, A.C. (1983) Establishment of the germ cell line in mammals. In Current Problems in Germ Cell Differentiation, eds. McLaren, A. & Wylie, C.C., pp41-69. Cambridge University Press.

Evans, E.P., Ford, C.E. & Lyon, M.F. (1977) Direct evidence of the capacity of the XY germ cell in the mouse to become an oocyte. Nature, **267**, 430-431.

Evans, C.W., Robb, D.I., Tuckett, F. & Challoner, S. (1982) Regulation of meiosis in the foetal mouse gonad. Journal of Embryology and experimental Morphology, **68**, 59-67.

Farias, E., Kajii, T. & Gardner, L.I. (1967) Technique for investigation of sex chromatin in amniotic membrane of rat foetuses. Nature, **214**, 499-500.

Fazel, A.R., Schulte, B.A., Thompson, R.P. & Spicer, S.S. (1987) Presence of a unique glycoconjugate on the surface of rat primordial germ cells during migration. Cell Differentiation, **21**, 199-211.

Francavilla, S. & Zamboni, L. (1985) Differentiation of mouse ectopic germinal cells in inta- and perigonadal locations. The Journal of Experimental Zoology, **233**, 101-109.

Godin, I., Wylie, C.C. & Heasman, J. (1990) Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture. Development, **108**, 357-363.

Grinsted, J., Byskov, A.G. & Andreasen, M.P. (1979) Induction of meiosis in fetal mouse testis in vitro by rete testis tissue from pubertal mice and bulls. Journal of Reproduction and Fertility, **56**, 653-656.

Grinsted, J. (1981) The relation between mesonephric kidney function and onset of meiosis in the mammalian ovary. In Development and function of reproductive organs., eds. Byskov, A.G. & Peters, H. pp13-27. Excerpta Medica.

Grinsted, J. & Byskov, A.G. (1981) Meiosis-inducing and meiosis-preventing substances in human male reproductive organs. Fertility and Sterility, **35**, 199.

Grinsted, J. & Aagesen, L. (1984) Mesonephric excretory function related to its influence on differentiation of fetal gonads. The Anatomical Record, **210**, 551-556.

Grinsted, J., Byskov, A.G., Christensen, I.J. & Jensenius, J.C. (1982) Influence of mesonephros on foetal and neonatal rabbit gonads I. Sex-steroid release by the testis in vitro. Acta Endocrinologica, **99**, 272-280.

Gubbay, J., Koopman, P., Collignon, J., Burgoyne, P. & Lovell_Badge, R. (1990) Normal structure and expression of Zfy genes in XY female mice mutant in Tdy. Development, **109**, 647-653.

Hadley, M.A., Weeks, B.S., Kleinman, H.K. & Dym, M. (1990) Laminin promotes formation of cord-like structures by Sertoli cells in vitro. Developmental Biology, **140**, 318-327.

Hahnel, A.C. & Eddy, E.M. (1986) Cell surface markers of mouse PGCs defined by two monoclonal antibodies. Gamete Research, **15**, 25-34.

Hahnel, A.C. & Eddy, E.M. (1987) The distribution of 2 cell surface determinants of mouse embryonal carcinoma and early embryonic cells. Journal of Reproductive Immunology, **10**, 89-110.

Hashimoto, N., Kubokawa, R., Yamazami, K., Noguchi, M. & Kato, Y. (1990) Germ cell deficiency causes testis cord differentiation in reconstituted mouse fetal ovaries. The Journal of Experimental Zoology, **253**, 61-70.

Heasman, J., Hynes, R.O., Swan, A.P., Thomas, V. & Wylie, C.C. (1981) Primordial germ cells of Xenopus embryos: the role of fibronectin in their adhesion during migration. Cell, **27**, 437-447.

Heath, J.K. (1978) Characterisation of a xenogeneic antiserum raised against the fetal germ cells of the mouse: cross reactivity with embryonal carcinoma cells. Cell, **15**, 299-306.

Heath, J.K. & Wylie, C.C. (1981) Cell surface molecules of mammalian foetal germ cells. In Development and Function of Reproductive Organs, eds. Byskov, A.G. & Peters, H. pp83-91. Amsterdam, Oxford, Princeton: Excerpta Medica.

Hilscher, B., Hilscher, W., Bulthoff-Ohnholz, B., Kramer, U., Birke, A., Pelzer, H. & Gauss, G. (1974) Kinetics of gametogenesis I. Comparative histological and autoradiographic studies of oocytes and transitional

prospermatogonia during oogenesis and prespermatogenesis. Cell and Tissue Research, **154**, 443-470.

Jeon, K.W. & Kennedy, J.R. (1973) The primordial germ cells in early mouse embryos: light and electron microscopic studies. Developmental Biology, **31**, 275-284.

Josso, N., Picard, J-Y. & Tran, D. (1977) The anti-Müllerian hormone. Recent Progress in Hormone Research, **33**, 117-167.

Josso, N., Vigier, B., Tran, D. & Picard, J.Y. (1985) Initiation of production of anti-Müllerian hormone by the fetal gonad. Archives d'Anatomie microscopique et de morphologie experimentale, **74**, 96-100.

Josso, N., Tran, D., Picard, J.Y. & Vigier, B. (1986) Physiology of anti-Müllerian hormone: in search of a new role for an old hormone. In Development and Function of Reproductive Organs, ed. Tsafriri, A., pp73-84. New York: Raven Press.

Jost, A. (1947) Recherches sur la différenciation sexuelle de l'embryon de lapin. III Rôle des gonades foetales dans la différenciation sexuelle somatique. Archives Anatamie Microscopique Morphologie Experimentale, **36**, 271-315.

Jost, A. (1953) Problems of fetal endocrinology: the gonadal and hypophyseal hormones. Recent Progress in Hormone Research, **8**, 379-418.

Jost, A. (1972) Donéés préliminaires sur les stades initiaux de la différenciation du testicule chex le rat. Archives d'Anatomie microscopique, **61**, 415-438.

Jost, A., Vigier, B. & Prépin, J. (1972) Freemartins in cattle: the first steps of sexual organogenesis. Journal of Reproduction and Fertility. **29**. 349-379.

Jost, A., Vigier, B., Prépin, J. & Perchellet, J.P. (1973) Studies on sex differentiation in mammals. Recent Progress in Hormone Research, **29**, 1-41.

Kitahara, Y. (1923) Uber die Entstehung der Zwischenzellen der Keimdrusen des Menschen und der Saugetiere und uber deren physiologische Bedeutung. Arch. Entwicklungsmech. Org., **52**, 571-604.

Kuwana, T., Miyayama, Y., Kajiwara, Y. & Fujimoto, T. (1987) Behaviour of chick PGCs moving toward gonadal primordium in vitro: scanning electron microscopic study., The Anatomical Record, **219**, 164-170.

Leivo, I., Vaheri, A., Timpi, T. & Wartiovaara, J. (1980) Appearance and distribution of collagens and laminin in the early mouse embryo. Developmental Biology, **76**, 100-114.

Lovell-Badge, R. & Robertson, E. (1990) XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. Development, **109**, 635-646.

Luciani, J.M., Deveictor, M. & Stahl, A. (1977)

Preleptotene chromosome condensation stage in human foetal and neonatal testes. Journal of Embryology and experimental Morphology, **38**. 175-186.

Mackay, S. and Smith, R.A. (1986) The differentiation of mouse gonads in vitro: a light and electron microscopical study. Journal of Embryology and experimenatal Morphology, **97**, 189-199.

Mackay, S. & Smith, R.A. (1989) Mouse gonadal differentiation in vitro in the presence of fetal calf serum. Cell Differentiation and Development, **27**, 19-28.

Magre, S. & Jost, A. (1984) Dissociation between testicular organogenesis and endocrine cytodifferentiation of Sertoli cells. Proceedings of the National Academy of Science. USA, **81**, 7831-7834.

Makabe, S. & Motta, P.M. (1986) Elimination of germ cells during differentiation of the human ovary: an electron microscopic study. European Journal of Obstetrics Gynecology and Reproductive Biology, **22**, 271-286.

McCoshen, J.A. & McCallion, D.J. (1975) A study of the primordial germ cells during their migratory phase in Steel mutant mice. Experientia, **31**, 589-590.

McGadey, J. (1970) A tetrazolium method for non-specific alkaline phosphatase. Histochemie, **23**, 180-184.

McLaren, A. (1981) Germ Cells and Soma: a new look at an

old problem, New Haven: Yale University Press.

McLaren, A. (1984) Meiosis and differentiation of mouse germ cells. Symposia of the Society for Experimental Biology, **38**, 7-23.

McLaren, A. (1985) Relation of germ cell sex to gonadal differentiation. In The Origin and Evolution of Sex, eds. Halverson, H.O. & Monroy, A., pp289-300.

McLaren, A. (1987a) Sex determination and H-Y antigen in mice. In Genetic Markers of Sex Differentiation, eds. Haseltine, F., McLure, P., Goldberg, M.E., pp87-97. New York:Plenum Press.

McLaren, A. (1987b) Testis determination and the H-Y hypothesis. In Current Topics in Developmental Biology, **23**, 163-183.

Mejbaum-Katzenellenbogen, W. & Dobryszycka, W.M. (1959) New method for quantitative determination of serum proteins separated by paper electrophoresis. Clinica Chimica Acta, **4**, 515-522.

Merchant, H., (1975) Rat gonadal and ovarian organogenesis with and without germ cells. An ultrastructural study. Developmental Biology, **44**, 1-21.

Merchant-Larios, H. & Alvarez-Buylla, A. (1986) The role of extracellular matrix and tissue topographic arrangement in mouse primordial germ cells migration. In Development and

Function of the Reproductive Organs. Serono Symposia Review no. 11., pp1-11, Ares-Serono Symposia.

Mintz, B. & Russell, E.S. (1957) Gene induced embryological modifications of primordial germ cells in the mouse. The Journal of Experimental Biology, **134**, 207-236.

Mitchell, P. A. & Burghardt, R.C. (1986) The ontogeny of nexuses (gap junctions) in the ovary of the fetal mouse. The Anatomical Record, **214**, 283-288.

Monk, M. & McLaren, A. (1981) X-chromosome activity in foetal germ cells of the mouse. Journal of Embryology and experimental Morphology, **63**, 75-84.

Müller, U., Singh, L., Grund, S., Jones, K.W. (1982) Ovarian cells participate in the formation of tubular structures in mouse/rat heterosexual gonadal co-cultures: a direct demonstration by in situ hybridisation. Differentiation, **22**, 136-138.

Müller, U. & Schindler, H. (1983) Testicular differentiation - a developmental cascade. Morphogenetic effects of H-Y antigen and testosterone in the male mammalian gonad. Differentiation Supplement, **23**, S99-S103.

O, W.S. & Baker, T.G. (1976) Initiation and control of meiosis in hamster gonads in vitro. Journal of Reproduction and Fertility, **48**, 399-401.

O, W.S. & Baker, T.G. (1978) Germinal and somatic cell

interrelationships in gonadal sex differentiation. Ann Biol anim Bioch Biophys, **18**, 351-357.

Odor, D.L. & Blandau, R.J. (1969) Ultrastructureal studies on fetal and early postnatal mouse ovaries. I. Histogenesis and Organogenesis. American Journal of Anatomy, **124**, 163-186.

Ozdzenski, W., Rogulska, R., Batakier, H., Brzozowska, M., Rembiszewska, A. & Stepinska, U. (1976) Influence of embryonic and adult testis on the differentiation of embryonic ovary in the mouse. Archives d'Anatomie Microscopique, **65**, 285-294.

Ozdzenski, W. & Presz, M. (1984) Precocious initiation of meiosis by male germ cells of the mouse. Archives d'Anatomie microscopique et de morphologie experimentale, **73**, 1-7.

Paranko, J., Pelliniemi, L.J., Vaheri, A., Foidart, J.M. & Lakkala-Paranko, T. (1983) Morphogenesis and fibronectin in sexual differentiation of rat embryonic gonads. Differentiation (Suppl.), **23**, S72-S81.

Patsavoudi, E., Magre, S., Castanier, M., Scholler, R. & Jost, A. (1985) Dissociation between testicular morphogenesis and functional differentiation of Leydig cells. Journal of Endocrinology, **105**, 235-238.

Picard, J.Y., Goulut, C., Bourrillon, R. & Josso, N. (1986) Biochemical analysis of bovine testicular anti-Müllerian

hormone. Febs Letters, **195**, 73-76.

Picon, R. (1969) Action du testicule foetal sur le développement in vitro des canaux de Müller chez le rat. Archives Anatomie Microscopique Morphologie Experimentale, **58**, 1-19.

Prépin, J., Charpentier, G. & Jost, A. (1985a) Action du testicule foetal sur le nombre des cellules germinales de l'ovaire du foetus de rat, in vitro. Comptes Rendus Académie des Sciences de Paris. Série III, **300**, 43-47.

Prépin, J., Gibello-Kervran, C., Charpentier, G. & Jost, A. (1985b) Number of germ cells and meiotic prophase stages in fetal rat ovaries cultured in vitro. Journal of Reproduction and Fertility, **73**, 579-583.

Prépin, J., Charpentier, G., Hida, N., Jost, A. & Maingourd, M. (1986) Isolement par liaison a des membranes d'ultrafiltration, d'un facteur testiculaire limitant le nombre de cellules germinales dans l'ovaire foetal de rat in vitro. Comptes Rendus Académie des Sciences. Série III, **303**, 123-126.

Prépin, J. & Hida, N. (1989a) Influence of age and medium on formation of epithelial cords in the rat fetal ovary in vitro. Journal of Reproduction and Fertility, **87**, 375-382.

Prépin, J. & Hida, N. (1989b) Limitation du nombre des cellules germinales dans l'ovaire foetal de rat par des

ovaires plus âges, in vitro. Comptes Rendus Académie des Sciences. Série III., **308**, 61-64.

Price, J.M., Donahoe, P.K., Ito, Y. & Hendren, W.H. (1977) Programmed cell death in the Müllerian duct induced by Müllerian inhibiting substance. American Journal of Anatomy, **149**, 353-376.

Regenass, U., Friedrich, T.D. & Stevens, L.C. (1982) Experimental induction of testicular teratomas in dissociated-reaggregated chimaeric gonads. Journal of Embryology and experimental Morphology, **72**, 153-167.

Rivarola, M.A., Podesta, E.J. & Chemes, H.E. (1972) In vitro testosterone-14C metabolism by rat seminiferous tubules at different stages of development: Formation of 5a-Androstenodiol. Endocrinology, **91**, 537-542.

Rogulska, T., Ozdzenski, W. & Komar, A. (1971) Behaviour of mouse primordial germ cells in the chick embryo. Journal of Embryology and experimental Morphlogy, **85**, 155-164.

Rojas, M.A., Morales, B. and Exponda, P. (1984) Foetal mieosis in the testis of the rodent Octodon degus. International Journal of Andrology, **7**, 529-541.

Rugh, R. (1968) The Mouse, its Reproduction and Development, Minneapolis: Burgess.

Russo, J. & de Rosas, J.C. (1971) Differentiation of the Leydig cell of the mouse testis during the fetal period -an

ultrastructural study. American Journal of Anatomy, **130**, 461-480.

Simpson, E., Chandler, P., Goulmy, E., Disteche, C.M., Ferguson-Smith, M.A. & Page, D.C. (1987) Separation of the genetic loci for the H-Y antigen and for testis determination on human Y chromosome. Nature, **326**, 876-878.

Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R. & Goodfellow, P.N. (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature, **346**, 240-244.

Snow, M.H.L. (1981) Autonomous development of parts isolated from primitive-streak-stage mouse embryos. Is development clonal? Journal of Embryology and experimental Morphology, **30**, 97-118.

Spiegelman, M. & Bennett, D. (1973) A light- and electron-microscopic study of primordial germ cells in the early mouse embryo. Journal of Embryology and experimental Morphology, **30**, 97-118.

Stein, L.E. & Anderson, E. (1981) In vitro analysis of ovarian differentiation and the initiation of meiosis in the rat. Acta. anat., **110**, 189-205.

Takahashi, M., Hayashi, M., Manganaro, T.F. & Donahoe, P.K. (1986) The ontogeny of Müllerian inhibiting substance in

granulosa cells of the bovine ovarian follicle. Biology of Reproduction **35**, 447-453.

Taketo, T. & Koide, S.S. (1981) In vitro development of testis and ovary from indifferent fetal mouse gonads. Developmental Biology, **84**, 61-66.

Taketo, T., Thau, R.B., Adeyemo, O. & Koide, S.S. (1984a) Influence of adenosine 3':5'-cyclic monophosphate analogyes on testicular organization of fetal mouse gonads in vitro. Biology of Reproduction, **30**, 189-198.

Taketo, T., Merchant-Larios, H. & Koide, S.S. (1984b) Induction of testicular differentiation in the fetal mouse ovary by transplantation into adult male mice. Proceedings of the Society for Experimental Biology and Medicine, **176**, 148-153.

Taketo-Hosotani, T., Merchant-Larios, H., Thau, R.B. & Koide, S.S. (1985a) Testicular cell differentiation in fetal mouse ovaries following transplantation into adult male mice. The Journal of Experimental Zoology, **236**, 229-237.

Taketo, T., Koide, S.S. & Merchant-Larios, H. (1985b) Gonadal sex differentiation in mammals. In The Origin and Evolution of Sex, pp271-287. Alan R. Liss.

Taketo, T., Seen, C.D. & Koide, S.S. (1986) Requirement of serum components for the preservation of primordial germ

cells in testis cords during early stages of testicular differentiation in vitro in the mouse. Biology of Reproduction, **34**, 919-924.

Taketo-Hosotani, T. (1987) Factors involved in the testicular development from fetal mouse ovaries following transplantation. The Journal of Experimental Zoology, **241**, 95-100.

Taketo-Hosotani, T. & Sinclair-Thompson (1987) Influence of the mesonephros on the development of fetal mouse ovaries following transplantation into adult male and female mice. Developmental Biology, **124**, 423-430.

Tam, P.P.L. & Snow, M.H.L. (1981) Proliferation and migration of primordial germ cells during compensatory growth in moue embryos. Journal of Embryology and experimental Morphology, **64**, 133-147.

Tavendale, S.J., Mackay, S. & Smith, R.A. (1987) Beneficial effects of fibronectin on isolated mouse primordial germ cells cultured in vitro. Journal of Anatomy, (abstract),**155**, 228.

Theiler, K. (1972) The House Mouse. Development and Normal Stages from Fertilisation to Four Weeks of Age,, Berlin, Heidelberg, New York : Springer Verlag.

Tung, P.S., Skinner, M.K. & Fritz, I. B. (1984) Cooperativity between Sertoli cells and peritubular myoid cells in the formation of the basal lamina in the

seminiferous tubule. Annales of the New York Academy of Science, **438**, 435-446.

Turner, CD. & Asakawa, H. (1964) Experimental reversal of germ cells in ovaries of fetal mice. Science, 1344-1345.

Ueno, S., Takahashi, M., Manganaro, T.F., Ragin, R.C. & Donahoe, P.K. (1989) Cellular localization of Müllerian inhibiting substance in the developing rat ovary. Endocrinology, **124**, 1000-1006.

Upadhyay, S., Luciani, J.M. & Zamboni, L. (1979) The role of the mesonephros in the development of indifferent gonads and ovaries of the mouse. Ann. Biol. anim Bioch Biophys, **19**, 1179-1196.

Upadhyay,S., Luciani, J.M. & Zamboni, L. (1981) The role of the mesonephros in the development of the mouse testis and its excurrent pathways. In Development and Function of Reproductive Organs, eds. Byskov, A.G. & Peters, H.

Upadhyay, S. & Zamboni, L. (1982) Ectopic germ cells: Natural model fro the study of germ cell sexual differentiation. Proceedings of the National Academy of Science. USA, **79**, 6584-6588.

Valentino, O., Agelopoulou, R. & Magre, S. (1985) Action d'un analogue de la proline (adice L-azétidine-2-carboxylique) sur la différenciation in vitro du testicule

foetal de rat. Comptes Rendus Academie des Sciences Paris. Série III, **301**, 225-232.

Vigier, B., Picard, J-Y, Tran, D., Legeai, L. & Josso, N. (1984) Production of anti-Müllerian hormone: another homology between Sertoli and Granulosa cells. Endocrinolgy, **114**, 1315-1320.

Vigier, B., Watrin, F., Matgre, S., Tran, D. & Josso, N. (1987) Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospectibe ovaries exposed to it in vitro. Development, **100**, 43-55.

Vigier, B., Watrin, F., Magre, S., Tran, D., Garrigou, O., Forest, M.G. & Josso, N. (1988) Anti-Müllerian hormone and freemartinism: inhibition of germ cell development and induction of seminiferous cord-like structures in rat fetal ovaries exposed in vitro to purified bovine AMH. Reproduction Nutrition and Dévelopement, **28**, 1113-1128.

Wachtel, S.S., Ohno, S., Koo, G.C. & Boyse, E.A. (1975) Possible role for H-Y antigen in the primary determination of sex. Nature, **257**, 235-236.

Wartenberg, H. (1978) Human testicular development and the role of the mesonephros in the origin of a dual Sertoli cell system. Andrologia, **10**, 1-21.

Wartenberg, H. (1989) Differentiation and development of the testes. In The Testis, Second Edition. eds. Burger, H. & de Kretser, D., pp67-117. New York: Raven Press.

Yding-Anderson, C., Byskov, A.G. & Grinsted, J. (1981) Partial purification of the meiosis inducing substance (MIS). In Development and Function of Reproductive Organs, eds. Byskov, A.G. & Peters, H., pp73-80. Amsterdam, Oxford, Princeton: Excerpta Medica.

Yoshinaga, K., Hess, D.L., Hendrickx, A.G. and Zamboni, L. (1988) The development of the sexually indifferent gonad in the prosimian, Galago crassicaudatus crassicaudatus (1988) The American Journal of Anatomy, **181**, 89-105.

Zamboni, L. & Merchant, H. (1973) The fine morphology of mouse priordial germ cels in extragonadal locations. American Journal of Anatomy, **137**, 299-335.

Zamboni, L. & Upadhyay, S. (1983) Germ cell differentiation in mouse adrenal glands. Journal of Experimental Zoology, **228**, 173-193.