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ON THE ENDOCRINE FUNCTION OF THE HUMAN GRAAFIAN FOLLICLE

By

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PREFACE

The object of this thesis was to obtain an understanding of the changing cyclical relationship which exists between the theca and granulosa cells of the isolated human Graafian follicle, invitro, with respect to its ability to produce the three basic classes of sex steroids, namely the androgens, oestrogens and progestins. Correlations of follicular steroid activity in relation to; follicular size, the stage within the reproductive cycle when follicular explantation took place, the distribution and activity of Δ^5 -3 β -hydroxysteroid dehydrogenase and incubation with gonadotrophins and prolactin was determined.

A satisfactory method of biopsy, microdissection and maintenance of whole human Graafian follicles and their isolated theca and granulosa cell components in organ culture was developed, with maintenance of both cellular integrity and enzyme activity as judged histologically and histochemically. These same follicles and their cellular components maintained good steroidogenic function and a predictable ability to respond to gonadotrophins for at least seven days, invitro.

DECLARATION

The experimental work described in this thesis was carried out in the Department of Obstetrics & Gynaecology, The Queen Elizabeth Hospital, Woodville, South Australia, from March 1974 to October 1977.

I hereby declare that these studies represent original work carried out by the author; where the author has availed himself of the work of others due acknowledgement has been made in the text. This dissertation has not previously been submitted in full or in part to any other University in application for any degree or diploma.

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I would particularly like to thank Dr. R.M. Moor for his advice regarding the incubation procedures, Dr. J.E.A. McIntosh for his help with the statistical analysis of the data, Regan Jeffries and Frederick Amato for their ever present willingness to dissect and incubate the Graafian follicles, Meredith Kaethner for setting up the radioimmunoassays for testosterone, oestradiol-17 β , progesterone and androstenedione, Leila Mac and Wendy Jones for performing many of the radioimmunoassay procedures, Dr. James Kirkland, Mr. Roy Ellis and staff of the Cytology and Gynaecological Pathology Laboratory for their skilled help and advice regarding both the histological and histochemical procedures, Mr. Kevin Crawshaw for performing the LH, FSH and prolactin radioimmunoassay procedures, Anne Dixon for the collection of much of the reference material and to all the staff of the Department of Obstetrics & Gynaecology for all their help and constructive criticism throughout the tenure of this work.

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This thesis is dedicated to my wife, Aileen whose unfailing support has been most valuable throughout the duration of this study.

NOMENCLATURESteroidsTrivial nameSystematic name

Cholesterol	Cholest-5-en-3 β -ol
Testosterone	17 β -Hydroxyandrost-4-en-3-one
Androstenedione	Androst-4-ene-3,17-dione
Oestradiol-17 β	Oestra-1,3,5(10)-triene-3,17 β -diol
Pregnene-1one	3 β -Hydroxypregn-5-en-20-one
17 α -Hydroxypregnenolone	3 β ,17 α -Dihydroxypregn-5-en-20-one
17 α -Hydroxyprogesterone	17 α -Hydroxypregn-4-ene-3,20-dione
Dehydroepiandrosterone	3 β -Hydroxyandrost-5-en-17-one
20 α -Hydroxyprogesterone	20 α -Hydroxypregn-4-en-3-one
5 α -Pregnanedione	5 α -Pregnane-3,20-dione
Oestrone	3-Hydroxyoestra-1,3,5(10)-trien-17-one
Oestriol	Oestra-1,3,5(10)-triene-3,16 α ,17 β -triol

* Oestrogen is used to denote oestradiol-17 β in this thesis.

Hormones

LH	Luteinising hormone
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotrophin

Nucleotides

cAMP	Cyclic Adenosine 3',5' - Monophosphate
ATP	Adenosine Triphosphate

Coenzymes

NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced NAD

Enzymes

3 β -HSD	Δ^5 -3 β -hydroxysteroid dehydrogenase
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Tetrazolium salt

N-BT	Nitro-Blue Tetrazolium
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Prostaglandins

PGF ₂ α	Prostaglandin F ₂ α
PGE ₂	Prostaglandin E ₂

SUMMARY

Two hundred and eight human Graafian follicles with diameters ranging from 3-12 millimetres were obtained from eighty nine ovarian biopsies, which were performed in women who had spontaneous, regular menstrual cycles. These follicles were incubated either as whole follicles in organ culture or dissected into their respective theca and granulosa cell components and incubated separately. In some instances gonadotrophins were included in the culture medium. The culture medium was aspirated daily and its content of testosterone, oestradiol-17 β and progesterone estimated by validated radioimmunoassay procedures. The follicles were either processed histologically for assessment of cellular morphology, theca and granulosa layer thickness and cell size, or were examined histochemically in an attempt to localize the site and activity of the enzyme, Δ^5 -3 β -hydroxysteroid dehydrogenase (3 β -HSD), at various times throughout the incubation period.

The following conclusions were drawn:-

1. The maintenance of isolated human follicles in organ culture to facilitate useful endocrine investigations has been achieved. Observations indicated that follicular growth was a continuum, but that rapid follicular growth occurred only during the late follicular phase of the reproductive cycle. In culture, endocrine function as assessed by steroid release was sustained but varied between individual follicles. This variation appeared related both to follicular size and the stage in the menstrual cycle when follicles were removed.

2. Follicles removed during the late follicular phase (preovular) of the cycle were significantly larger than follicles removed during the early follicular phase, luteal phase or the immediate post partum period. No significant difference in size could be demonstrated in follicles removed during the early follicular or luteal phase of the menstrual cycle. Follicles removed during the early post partum period were significantly smaller than both the luteal and the early follicular phase follicles.

3. On a weight for weight basis steroid activity in late follicular phase follicles appeared to be related to the size of the follicle. The release of oestrogen and progesterone increased progressively with increasing follicular size and approaching ovulation. A steady rise in testosterone release occurred until the late follicular phase follicle

reached a diameter of 8 mm, and then declined, coincident with a progressive increase in oestrogen and progesterone release. This change in steroid activity coincided with the appearance of pronounced 3β -HSD activity in the granulosa cell layer but with persistence of 3β -HSD activity in the theca layer.

4. Invitro, the smaller early follicular phase follicles released testosterone predominantly, and 3β -HSD activity mainly was confined to the theca interna cells. Luteal phase follicles also released similar amounts of androgen but less amounts of oestrogen and progesterone than late follicular phase follicles and retained moderate activity of 3β -HSD in both theca and granulosa cells. Follicles removed within the first week of parturition maintained steroid activity, which was comparable with that of unstimulated late follicular phase follicles, and also possessed moderate 3β -HSD activity in both the theca and granulosa cells.

5. Invitro, the steroid release by isolates of theca and granulosa cells obtained from late follicular phase follicles, indicated that the theca was the primary source of testosterone synthesis and the granulosa cells the primary source of progesterone synthesis. Interestingly, isolates of both theca and granulosa cells were able to produce oestrogen, indicating that both cell types may contribute to oestrogen synthesis within the follicle, thereby lending support to the two cell theory for optimal oestrogen production.

6. Small slices of postmenopausal ovary which were devoid of follicles released small amounts of testosterone and progesterone but no oestradiol- 17β . Histochemical localization for 3β -HSD in these ovaries demonstrated small patches of positive reaction indicating focal areas of steroid activity.

7. The incubation of follicles with gonadotrophins indicated that follicular phase follicles develop an ability to respond to FSH for both oestrogen and progesterone production, but that the luteal phase follicles tended to maintain their capacity to respond to FSH for increased progesterone production (a reflection of granulosa cell activity) but not for increased oestrogen production (a possible indication of failing theca cell aromatase activity). Incubation with HCG was associated with an increased progesterone output in both follicular and luteal phase follicles, increased testosterone output by follicular phase follicles but at the same time a decrease in the oestrogen production

by these follicles. This observation was consistent with the hypothesis that HCG may inhibit aromatase activity in the follicular phase follicle. The luteal follicle theca cells lost their ability to produce an increased release of oestrogen and testosterone following incubation with either FSH or HCG, however morphologically they did not regress as do the theca cells of the corpus luteum. Although post partum follicles produced steroids, invitro, they failed to respond to FSH apart from a transient increased output of progesterone. This observation was consistent with the hypothesis that FSH receptor sites were either sparse or non functional in these follicles.

8. Preliminary observations suggested that the addition of high concentrations of prolactin to the incubation media of whole follicles did not augment steroidogenesis and may well have an inhibitory effect, even in the presence of FSH.

9. Preliminary observations also suggested that human ovarian follicles were able to release relaxin, invitro. Maximum release of relaxin occurred from those follicles explanted during the luteal phase and in particular from the follicles removed from the ovary containing the corpus luteum. This hormone may be involved in the corpus luteum and in follicle regression or as a mediator of collagenase activity.

10. Collagen was demonstrated in the theca interna but was more marked in the theca externa of follicles from 2 to 12 mm in diameter. Collagen deposition was more abundant as the follicles became larger and was also present in luteal phase follicles. Collagen deposition was dispersed in the theca cells of the corpus luteum but was present in abundance in the surrounding stroma. The basement membrane lying between the theca and granulosa cells of follicles was prominent and stained identically to that of collagen. Evidence of the basement membrane or its remnants were not seen in corpora lutea. The circumferential pattern of collagen surrounding follicles probably appeared more prominent as the follicles grew due to a condensation effect on the surrounding connective tissue.

11. The combination of anaesthesia and surgery was associated with a significant elevation of blood prolactin levels. However follicular fluid prolactin levels obtained during surgery appeared to be independent of blood levels over the time interval studied.

PUBLICATIONS AND PRESENTATIONS ARISING FROM AND RELATED TO WORK
SUBMITTED IN THIS THESIS

1. Kerin, J.F.P., The Application of Tissue Culture to the Investigation of Human Ovarian Function. Presented before the Australian Congress of the Endocrine Society of Australia, August, 1974, and awarded the Nimmo Research Prize.
2. Kerin, J.F.P., Moor, R.M. and Seamark, R.F., Human Graafian Follicles in tissue culture : Correlations between oestrogen and progesterone production and the site of Δ^5 - 3β -hydroxysteroid dehydrogenase activity. Presented at the Australian Society for Reproductive Biology Proceedings, Brisbane, August 1976, and published in Theriogenology (1976), 6, 605.
3. Kerin, J.F.P., Seamark, R.F. and Cox, L.W., Steroid Profiles of Human Ovarian Follicles in Organ Culture. Presented at the 21st British Congress of Obstetrics & Gynaecology, Sheffield, July, 1977, by L.W. Cox.
4. Kerin, J.F.P. and Seamark, R.F., Steroid Production by Isolated Human Theca & Granulosa Cells in Tissue Culture. Presented at the Australian Society of Reproductive Biology Proceedings, Melbourne, August, 1977.
5. Kerin, J.F.P., Seamark, R.F. and Cox, L.W., Steroid Release by Human Ovarian Follicles and Ovarian Tissues in Culture. Submitted to the Journal of Endocrinology for assessment, November, 1977.
6. Kerin, J.F.P., Matthews, C.D. and Svigos, J.M., (1976) Linear and Quantitative Migration of Stored Semen through Cervical Mucus during the Periovular Period. Fertility and Sterility, 27, 1054-1058.
7. Kerin, J.F.P., Matthews, C.D., Svigos, J.M. and Makin, A., Migration of Stored Human Spermatozoa through Cervical Mucus in relation to the Preovulatory LH Surge. Presented at the Annual Meeting of the Australian Society for Reproductive Biology in Sydney, August, 1975, and accepted for publication in the Journal of Reproductive Fertility, (1976), 46, 499-500.
8. Kerin, J.F.P., Matthews, C.D., Hopkins, R.E. and Svigos, J.M., Cervical Mucus & Hormonal Profiles in Conceptual Cycles of Women undergoing Donor Artificial Insemination. Presented at the Obstetrics & Gynaecology Research Society Meeting, Melbourne, August, 1977, and accepted for publication in the Aust. & N.Z. J. of Obstet. & Gynaec.
9. Kerin, J.F.P. Cervical factors in infertility: Artificial insemination. Accepted for publication in. Modern Medicine, March, 1978.



CHAPTER ONE

INTRODUCTION

1.

1.1 RATIONALE FOR THE STUDY

A better understanding and treatment of the subfertile woman has been achieved in the last two decades. The multi-centred control needed to regulate the complex hormonal environment of the ovarian hypothalamic pituitary axis in order to achieve a potentially conceptual reproductive cycle is still poorly understood. From working in an ovulation induction and an artificial insemination clinic, while undertaking this study during the past three years, it has become increasingly obvious that there are more subtle defects contributing to subfertility in a number of women with either induced or spontaneous ovular cycles. Cox, (1975, 1977), has noted that 15% of women who undergo successful ovulation induction and are otherwise potentially fertile, fail to conceive after regular intercourse during the peri-ovular period, when partnered with a potentially fertile male. In patients having spontaneous ovular cycles, favourable cervical mucus, an accepted normal FSH, LH, oestradiol-17 β and prolactin blood levels, together with a thermal shift on a basal temperature chart, only 55% conceive after insemination during the periovular period, using good quality semen (Kerin, 1977). The semen was assessed by the criteria of Eliasson, (1971), and by its ability to migrate through periovular cervical mucus, (Kerin, Matthews, Svigos, Makin, Symons and Smeaton, 1976). It must be accepted that there could be multiple extra ovarian factors contributing to failure to conceive, but it is highly likely that delicate intraovarian malfunctions may be key factors in some circumstances. The concept of 'poor' and 'good' ovulations is reflected in the clinical terms of follicular phase and luteal phase insufficiency. A study of 18 conceptual cycles was undertaken (Kerin, Matthews, Hopkins and Svigos, 1977), and the parameters above examined. All the parameters as judged by accepted standards for each were within the normal to high range of normality. A current study comparing the conceptual parameter ratings against the non-conceptual parameter ratings is being undertaken by a fellow colleague, Dr. R. Hopkins. Preliminary results indicate that significant differences may be present.

Despite the high success rate of ovulation induction, many of these women revert back to acyclic, non ovular ovarian function, indicating primary intraovarian malfunction. It is therefore unlikely

that much progress will be made towards restoring spontaneous cyclical function in women with disorders of ovarian function until more is known about the regulators of follicular maturation. It is the follicle which 'nurses' the ovum from foetal life to ovulation, and presumably the 'healthy' follicle that is destined to mature into the healthy corpus luteum, which in turn potentially provides the ideal conditions for gestation. The work of Knobil, (1974) and Yen, (1975) have raised the status of the ovary by producing considerable experimental evidence which implicates it as the site of inherent cyclical control of hypothalamic pituitary function. Most of the work in this thesis could not have been achieved without the radioimmunoassay techniques employed which were both sensitive and specific for a particular hormone. This thesis centres around an attempt to critically examine factors controlling human ovarian follicle behaviour, in the hope of achieving a better understanding of female ovarian function.

1.2. HISTORICAL REVIEW

Observations related to the human ovary, 1555 - 1950

The original name for the ovary was the female testis and dates from the time of Herophilus (300 B.C.). In the second edition of his "De Fabrica", Versalius (1555), remarks upon the irregular surface of the "testes muliebres". The name, "ovarium", appears to have been introduced early in the seventeenth century, followed by the discovery of ovarian follicles and 'ova' some years later, (De Graaf, 1668).

Richard Lower (1670), suggested that a serum issued into the ventricles of the brain and out of them through the infundibulum of the glandula pituitaria, was not distilled upon the palate, but formed again into the blood and mixed with it. It is generally accepted that the first genuine belief in the existence of an 'internal secretion' was voiced by Théophile de Bordeu in 1775, who suggested that each organ of the body gave off emanations which were necessary and useful to the whole body. The actual term 'internal secretion' was first employed by Claude Bernard in 1855 in a lecture at the College de France, and to him, the credit is given as the founder of the modern conception of internal secretions. Early in the present century William Bayliss and E.H. Starling were dissatisfied with the term 'internal secretion' and in search for a better one, suggested the word 'hormone'. Starling, in the Croonian Lectures at the Royal College of Physicians in 1905 used the phrase 'these chemical messengers or Hormones', derived from the Greek word meaning, I excite or I arouse. The terms 'endocrine' and 'endocrinology', also derived from the Greek, come into use later in this century.

The identification of the human ovum is generally credited to Ernest V. Baer who described it in 1827. The first factual discovery of the physiology of the sex organs and their hormones should be credited to Berthold (1849). He showed that the transplantation of the cocks' testes into some other part of the body, prevented the atrophy of the cock's comb which usually followed castration. In 1868, a 'wave or tide theory' was developed. This theory was based on the assumption that a woman's vital activity, as shown by her pulse rate, temperature and blood pressure, were governed by a 'wave' or 'tide', whose length corresponded to that of the intermenstrual interval.

Aveling, (1874) had come as near the truth as anyone when he declared that ovulation preceded menstruation and that a menstrual

decidua was built up each month for the nidation of a fertilized ovum. Furthermore he went on to say that if nidation was not forthcoming, a degenerative process of 'denidation' followed, of which the menstrual flow was the outward visible sign. This deduction was in line with Power's earlier dictum that, 'women menstruate because they do not conceive'. At this time it was thought that the stimulus to menstruate came, via 'an ovarian nerve', from the tension within the ovary caused by the ripening follicle. The corpus luteum was neglectfully regarded as merely the end product of the Graafian follicle.

Both Pozzi (1893) and Webster (1898) give the ovary with a detailed outline of its anatomy, physiology and pathology, first place in their books entitled, 'A Treatise on Gynaecology, Clinical and Operative,' and 'Diseases of Women', respectively. In Pozzi's text it was noted that Nagel found the primordial ovum to measure 50 to 70 μ in diameter and its nucleus 29-32 μ in diameter. Sutton, (1897) and Heap, (1898), suggested that there was a possible analogy between menstruation in women and heat or rut in lower animals and that in some way both functions were connected with Nature's preparations for a possible impregnation. Comte (1898) noted an increase in size of the pituitary gland during pregnancy. Fasbender, (1900), ascribed the fallopian tube as the meeting place for ovum and spermatozoa and described the development of the uterine bed. In 1903, Fraenkel produced evidence to show that the corpus luteum was really a gland whose internal secretion was essential to the development of the decidua, the maintenance of early pregnancy and the occurrence of menstruation.

Prior to the epoch making work of Hitschmann and Adler in 1908, it was thought that the mucous lining of the uterus was a thin membrane of stable structure, subject only to some swelling and congestion immediately before menstruation. During the last half of the eighteenth century, based largely upon the work of Negrier, Gendrin and Pouchet in France and Pflüger's work in Germany, it became apparent that ovulation and menstruation were causally related. Hitschmann and Adler, (1908), showed that in the endometrium there was a regular cycle of changes associated with menstruation. They noted that the premenstrual endometrium was very similar in appearance to the decidua of pregnancy. It was not long before they deduced that these changes were post-ovulatory in time and pre-gestational in hopeful purpose, and it became clear that the decidua of pregnancy was just an intensification of them once that purpose had been achieved. It was later shown that only an endometrium sensitized by the progestogenic hormone of the corpus luteum

would cause a fully developed decidual reaction affording the fertilized ovum the best possible environment. The recognition of the fact that there was both an endometrial and ovarian cycle, led to an immense amount of research, which ultimately revealed the chronological correlation of the two. It soon became clear that ovulation occurred about the middle of the intermenstrual interval, and not during or immediately after the menstrual flow as had been thought. At this time an intensive search was begun for what came to be called, 'sex hormones', which culminated in the discovery, and ultimately the isolation of oestrogenic and progestogenic hormones of the ovary, and the proof that they were responsible for the endometrial changes.

Cushing, (1906), demonstrated that partial excision of the pituitary caused hypo-pituitarism with the clinical picture of Adiposogenital Dystrophy. In 1908 he came to the conclusion that the opposite condition, acromegaly, was due to excessive pituitary action. In 1919, Blair Bell, published his famous monograph, entitled 'The Pituitary', which gave a detailed account of its anatomy, physiology and function up to that time. These observations set the stage for later workers to establish the relationships between the ovary and pituitary gland.

Bryce, Teacher and Munro Kerr, (1908), and Young (1911) described the human ovum and first described the way in which the human blastocyst burrowed down into the substance of the decidua; the reaction of the decidua to this invasion, and the way in which the maternal circulation first comes into relation with the zygote before the formation of the intervillous space. Fraenkel, (1911), showed that if the rabbit corpus luteum was destroyed in pregnant rabbits, abortion almost invariably followed (Eden and Lockyer, 1928). In 1912, Adler produced the changes of oestrus by injecting into virgin animals, watery extracts of ovary.

Stockard and Papanicolaou, (1917), noted that the vaginal epithelium of rodents became cornified at the onset of oestrus. The oestrogenic effect of extracts containing oestrogen was noted to occur on the vaginal epithelium of ovariectomized animals. It was recognition of this fact by Allen and Doisy in 1923, that gave origin to their test for the assessment of the activity of oestrogens. In 1917, Hammond, showed that the ovum can be fertilized within hours following ovulation in farm animals, and that spermatozoa lose their power of fertilization long before they lose their motility. It was noted by Grosser, (1927), that unless the fertilized ovum had embedded at least two days before menstruation was due, menstruation would not be arrested and the ovum

swept away in the menstrual flow.

In the 1920's dating of young corpora lutea was established, from which the time of ovulation was deduced as occurring between the 10th and 17th days after the start of the last menstrual period. Parkes and Bellerby, (1926), successfully extracted a hormone they called oestrin from the ovary using fat solvents. Other names for this hormone at the time were folliculin and theelin.

Aschheim and Zondek, (1926), discovered the gonadotrophic activity of the anterior pituitary and that the urine of pregnant females contained a urinary gonadotrophin with a similar action to that possessed by the gonadotrophic principle of the anterior pituitary. They maintained that there were two anterior pituitary gonadotrophic hormones, Prolan A, which stimulated the ovary to ripen ovum bearing follicles, and Prolan B, which produced luteinizing effects on these follicles. The follicle stimulating principle was prepared from the serum of pregnant mares, and luteinizing hormone from pregnancy urine. In 1928, Aschheim and Zondek published their test for pregnancy based on the presence in the urine of pregnant women, 'gonadotrophic hormones' which stimulate the ovary to secrete its own hormones. The gonadotrophic hormones were found to be identical with the hormone of the anterior lobe of the pituitary. Thus the primacy of the pituitary gland over the female reproductive physiology was established. The pituitary was later found to be controlled by the hypothalamus via gonadotrophic releasing hormones (gonadoliberins). However recently experimental evidence indicates that in many species, including man, the hypothalamic-pituitary system is essentially acyclic and that the ovary itself is the seat of inherent cyclic function (Knobil, 1974; Yen, 1975).

Hartman, (1929), described the granulosa cells as 'nurse cells' in that their relationship to the ovum was a supportive and nutritional one. The mechanism of ovulation was poorly understood. Strassman, (1938), proposed that the growing Graafian follicle ascends to the ovarian surface by following the line of least resistance which was provided by an 'eccentric' theca interna growth cone of cells. He found that this growth cone, wedge shaped in section, pointed towards the surface of the ovary. Philipp, (1930), showed that the gonadotrophic principle found in urine originated from chorionic tissue of pregnancy. Ovulation was successfully induced in the human by the administration of gonadotrophic hormone obtained from pregnant mare's serum (Siegler, 1939).

In 1939, it was noted that only 400 follicles at the most are destined to ovulate in a woman's lifetime, and that those that did not ovulate undergo a process of retrogression termed follicle atresia, the first signs of which are seen in the ovum, which undergoes hyaline and fatty change. The granulosa cells regress followed by those of the theca interna, the follicle being replaced by hyaline tissue to form a corpus atretica. It was thought that surviving theca cells went on to form the interstitial cells of the ovary (Schlink, Chapman, Stenning and Chenhall, 1949). It was felt that waves of follicles mature and then regress with a rhythm fundamentally related to the ovulation cycle and that during pregnancy this rhythm continued, not to ovulation, but underwent degeneration. This theory has recently been questioned by Turnbull, Braden and Mattner (1977) with regard to the ovine ovary.

To sum up, the level of reproductive endocrinology and hormonal therapy to 1950 can be summarized as follows:-

- (1) The action of oestrogens on the uterus, vagina and breasts had been characterized.
- (2) The cyclical blood levels of oestrogen and progesterone in relation to the menstrual cycle had been established.
- (3) The basic hypothalamic-pituitary-ovarian axis had been defined.
- (4) The relationship between ovarian steroid failure and high levels of circulating gonadotrophins post menopausally had been related to menopausal symptomatology.
- (5) Synthetic oestrogens and progestogens were available and being used therapeutically with limited success, for such conditions as abnormal uterine bleeding, suppression of lactation, menopausal symptoms, pituitary insufficiency, amenorrhoea, dysmenorrhoea, atrophic vaginitis and threatened abortion.

Even in 1950, according to Kerr, Johnstone and Phillips, the study of endocrinology was a science in the hands of a number of serious minded research workers, but the practice of endocrine therapy, at least as far as the sex hormones was concerned, was not a scientific undertaking, but a highly commercialized and very profitable business in the hands of a regrettably large number of practitioners. As early as 1940, eminent authorities like Munro Kerr and Novak wondered if the prolonged and heavy use of oestrogens may act as carcinogenic agents.

A short tribute to Regnier De Graaf

Regnier De Graaf (1668), holds a unique position in the history of reproductive biology. He stands at the summit of the achievement of the older anatomists, whose work he summarized, corrected and extended. He was one of the first to take advantage of the microscope developed by Leuwenhoeck and stands at the beginning of modern microscopical anatomy. De Graaf advanced the knowledge regarding the anatomy and physiology of both male and female reproductive tracts, by meticulous anatomical dissection and astute deductions regarding each organ's function. He described corpora lutea and ascribed to the fallopian tubes their true function. He was not the first to describe Graafian follicles to which his name is deservedly attached, but he did describe more accurately how they changed and developed. He did not distinguish the ovum from the follicle, however he did record the fact that the youngest fertilized ovum, found in the oviduct was considerably smaller than the vesicle; a remarkable achievement at the time using very primitive microscopes (translation by Jocelyn and Setchell, 1972).

Most of the material for the historical review was obtained from books by Pozzi (1893), Webster (1898), Eden (1928), Lane-Roberts (1939), Robson (1940), Siegler (1944), Schlink (1949) and Munro Kerr (1954). It was felt that the names and dating of these important advances in reproductive endocrinology should be noted despite the failure to sight the original articles in many instances.

1.3. RECENT LITERATURE REVIEW 1950-1977

The Graafian Follicle

The ovarian follicle is a major ovarian compartment which enables the ovary to fulfill its dual function of gametogenesis and steroidogenesis. Many of the early experiments designed to study the steroid activity of the various ovarian tissues, were performed on a variety of animals. It has become evident that there are marked species differences with respect to both enzyme and steroid activity within various intraovarian compartments and the different cells within these compartments. Species ranging from the domestic fowl, (Senior and Furr, 1975), the rat, (Oakley and Stitch, 1967; Fortune and Armstrong, 1977), the sheep (Moor, 1973; Hay and Moor, 1973 & 1975; Baird, Baker, McNatty and Neal, 1975), the monkey (Channing, 1976), the cow (Lacroix, Eechaute and Leusen, 1974), the pig, (McGaughey, 1977), and the human (Ryan, 1967

Channing 1969), have been extensively investigated and the differences noted with respect to steroid and enzyme location and activity.

1.3.1. Elucidation of the steroid pathways in the Graafian follicle

Falk, (1959), studied the site of oestrogen production in the rat ovary using microtransplants. He found that oestrogen secretion only occurred when theca interna cells were transplanted in combination with granulosa cells. He concluded that this two-cell system was necessary to oestrogen production. Allen, (1941), demonstrated that follicular fluid, follicle wall and human corpus luteum contained oestrogen and that the follicular epithelium was the primary source of oestrogen. Zondek and Ashheim, (1931), had shown that after removal of the granulosa cell layer, the follicle wall still contained oestrogen, however they were unable to demonstrate oestrogen activity in the granulosa cells. Zondek (1931) also believed that the granulosa and corpus luteum cells in the human ovary secreted both progesterone and oestrogen (Falk, 1959).

Intensive research and major advances were made into the understanding of ovarian function in the early and mid 60's. The mechanism of steroid biosynthetic pathways was determined, (Ryan, 1967), the role of ovulation induction with gonadotrophins was advanced, (Gemzell, 1965) and steroid disorders related to ovarian disease states (Axelrod, 1962). It was noted that the fundamental steroid patterns were displayed by all steroid producing endocrine organs (Speroff, Glass, Kase, 1973). For instance the ovary differed from the testis in its functional capacity with respect to certain enzyme activity and subsequent steroid production, and from the adrenal gland in that it was deficient in 21-hydroxylase and 11 β -hydroxylase enzymes for the synthesis of glucocorticoids (cortisol and corticosterone) and mineralocorticoids (aldosterone and 11-deoxycorticosterone). Invitro studies on isolated human theca and granulosa cells, by Ryan and Petro, (1966), indicated that the different steroid activity between the granulosa and theca cells was a quantitative rather than a qualitative one. They suggested that the granulosa cells were deficient in 17-hydroxylase and 17, 20, desmolase activities and tended to metabolize pregnenolone via the Δ^4 -ketone pathway and consequent progesterone production; a characteristic of luteal tissue. The theca cells on the other hand had a strong 17-hydroxylase and desmolase enzyme system and directed the conversion of pregnanolone to androgens via the Δ^5 -hydroxyl pathway. Ryan and Short, (1965) demonstrated that equine granulosa cells had a greater ability than

theca cells to aromatize androgens, and thought that because of the granulosa cell layers avascularity prior to ovulation, its ability to synthesize steroids was limited. Lacroix et al, (1974) have shown that both the theca and granulosa cells of cow follicles convert pregnenolone to androstenedione predominantly through the Δ^5 -hydroxyl pathway and that the conversion of androstenedione to oestrogens was mainly carried out by the granulosa cells. However incubations of intact theca and granulosa yielded the highest amounts of oestrogen.

1.3.2. Factors controlling maturation of the Graafian follicle

(1) The pattern of follicular growth

Follicular growth has been described as a continuum, which goes on through foetal life, childhood, reproductive life, uninterrupted by pregnancy or other periods of nonovulation, (Peters, Byskov, Himelstein-Braw and Faber, 1975). Each day of the cycle follicles begin to grow and once started, grow continuously until they become atretic or ovulate, (Pedersen, 1970). Govan, (1968, 1970), has described the presence of small and large antral follicles throughout gestation in the human. There is therefore a sharp distinction between the continuum of follicular growth and the cyclic event of ovulation. The beginning of follicular growth appears to be regulated by intraovarian mechanisms and only in the final stages of maturation do the pituitary gonadotrophins play a major role in the mechanism of ovulation.

A study of follicular growth patterns in the ewe, by Turnbull, Braden and Mattner, (1977), demonstrated that follicles less than 1 mm in diameter rarely underwent atresia, the greatest incidence being in follicles of 1.5 - 2.5 mm in diameter. The growth rate of the granulosa layer was made by a calculation based on their mitotic index and mitotic time. Assessment of follicular growth in relation to diameter throughout the oestrus cycle also supported the concept of an asynchronous growth pattern. Treatment of ewes with PMS gonadotrophin maximally stimulated growth of follicles with a diameter greater than 3.5 mm, whereas followup treatment with HCG induced luteinization of most follicles greater than 3.5 mm, and induced atresia in small follicles of 1 - 3.5 mm in diameter. Studies on the cycling Rhesus monkey by Goodman, Nixon, Johnson and Hodgen, (1977), have indicated that by the mid follicular phase, the follicle destined to ovulate had been selected, and that no other follicles were soon competent to mature. Ablation of the preovular follicle did not alter

subsequent follicular growth or cyclical function.

Baird, Baker, McNatty and Neal, (1975), suggested that the variation in length of the follicular phase in many species of mammals may be related to the cellular origin of oestradiol secreted during the luteal phase. For instance, in the sheep where the only source of oestradiol was the Graafian follicle, follicular development proceeded unimpaired throughout the luteal phase, and so the 'follicular phase' which involved only the final stages of maturation of the Graafian follicle was relatively short. In primates, however, in which there was an extra follicular source of oestrogen from the corpus luteum, the secretion of gonadotrophins was suppressed during each luteal phase to a level too low to initiate and maintain follicular development. Therefore at the end of each luteal phase and the beginning of the subsequent follicular phase, it was necessary to initiate the growth of a new crop of small follicles. The time taken for these follicles to develop into preovulatory follicles determined the length of the follicular phase.

(2) The oocyte

El-Fouly, Cook, Nekola and Nalbandov, (1970), demonstrated that removal of the oocyte from the preovulatory follicle of the rabbit resulted in luteinization of the granulosa cells and the secretion of progesterone. This luteinizing inhibiting effect could also be overcome by the addition of LH to the follicles. It was suggested that as the oocyte matures it loses this inhibitory effect and by the time that the LH surge occurs just prior to ovulation, it can no longer suppress luteinization of the follicle, or more specifically, the granulosa cells. Hillensjö, DekeI and Ahrén, (1975), demonstrated that the preovulatory surge of gonadotrophins leads to the resumption of oocyte meiosis in rat follicles and morphological changes in the cumulus oophorus.

McGaughey, (1977), demonstrated that the addition of oestradiol-17 β , to denuded pig oocytes, invitro, inhibited their maturation which was overcome by either including progesterone in the medium or culturing the inhibited oocytes in steroid free medium. Also, oocytes cultured in media containing both oestrogen and progesterone, showed a higher incidence of normal meiotic maturation than control oocytes, cultured in media containing only oestrogen or progesterone.

(3) Development of receptor sites within the follicle

Lunenfeld, Kraiem and Eshkol, (1975), suggested that the number of

gonadotrophin receptors on granulosa and theca cells increased with follicular maturation and that steroids secreted by a family of follicles at a particular time modulated follicular growth by local intraovarian mechanisms. Channing and Kammerman, (1973), have shown that granulosa cells harvested from large pig follicles bind more HCG than adjacent small ones and may explain why large follicles tend to spontaneously luteinize in culture, and small follicles even in the presence of HCG do not, (Channing, 1970).

Nimrod, Erickson and Ryan, (1976), were able to demonstrate specific high affinity FSH receptor sites in rat granulosa cells, and by comparison the rabbit granulosa cells have low affinity FSH receptor sites; an interesting observation when one considers that the rat is a spontaneous ovulator like the human, whereas the rabbit is a reflex ovulator. Stouffer, Tyrey and Schomberg, (1976), have shown that porcine granulosa cells contain a homogeneous class of LH receptors whose number, but not affinity, increases during follicle maturation *in vivo*. Loss of receptors occurred *in vitro* after 48 hours associated with a drop in progesterone production, implying that an unknown factor responsible for the maintenance and development of the receptor population was missing. This is an important consideration, therefore, when incubating follicles *in vitro*, with gonadotrophins for extended periods of time.

1.3.3. The role of Prolactin in the Graafian follicle

McNatty, Sawers and McNeilly, (1974), reported that the production of progesterone by human granulosa cells *in vitro* required low physiological concentrations of prolactin whereas high concentrations were inhibitory and that this finding could partly explain why women with galactorrhoea, and high prolactin levels were anovular and amenorrhoeic. According to Goldenberg, Vaitukaitus and Ross, (1972), the growth of the Graafian follicle was maintained by the high concentration of oestradiol within the follicle which sensitized the cells to both FSH and LH. McNatty, Hunter, McNeilly and Sawers, (1975) noted that large human follicles of more than 8 mm in diameter, present in the late follicular phase, contained high concentrations of progesterone, oestradiol, low physiological levels of prolactin and relatively high levels of LH and FSH, and that one or more of these follicles was destined for ovulation.

1.3.4. Oestrogen and aromatase activity within the Graafian follicle

McNatty and Sawers, (1975), noted that mitotic activity and progesterone production of human granulosa cells, *in vitro*, only

occurred in follicles containing FSH and high concentrations of oestradiol. However mitosis could be induced invitro after the addition of FSH and oestradiol, provided the concentration of LH was low. It was found that premature exposure of granulosa cells to LH inhibited both mitotic activity and the steroidogenic potential of these cells. However once the preovular follicle had reached maturity, with its full compliment of granulosa cell LH receptor sites, it would respond to physiological levels of LH and prolactin (McNatty, et al., 1974), to produce progesterone.

Moon, Dorrington and Armstrong, (1975), were able to show that FSH stimulated aromatization of androgens to oestrogens in rat ovaries invitro and that granulosa cells in tissue culture also had this ability, (Dorrington, Moon and Armstrong, 1975). This led Baird, (1972), to investigate if androgens left the theca cell before aromatization in the sheep. By infusing sheep with anti-androgen antiserum, oestradiol levels were depressed. Since there was not total inhibition of oestrogen secretion by androgen antibodies, he suggested that a proportion of androgen was converted to oestrogen locally within the cell, which would be in keeping with Ryan's observation (1968), that both theca and granulosa cells have the ability to synthesize oestrogens invitro. These investigations gave support to the thesis that androgen precursors left the follicle (theca or granulosa) before aromatization invivo. It was suggested that androgens synthesized by the theca interna, left the cell and traversed the extra cellular space and basement membrane before being converted to oestrogens by the granulosa cells under the influence of FSH (Dorrington et al., 1975). Follicles in which the granulosa cells had been activated by FSH, had the ability to aromatize androgens and thus protect themselves from atresia, induced by high local androgen concentration (Baird, 1977). Hillier, Knazek and Ross, (1977), have shown that androgenic stimulation of progesterone production in preantral granulosa cells of rat follicles invitro, is modulated by the presence of oestrogen, and may be an important factor governing intraovarian control of follicular maturation invivo.

1.3.5. The effect of LH on aromatase activity

Seamark, Moor and McIntosh, (1974), were able to measure substrate accumulation after the addition of LH to sheep follicles in culture. The findings indicated that inactivation of first the aromatase system and then the desmolase system limited steroid production via the Δ^5 -hydroxyl pathway in the theca interna. As noted by Hay and Moor, (1973), this

treatment induced morphological luteinization of the granulosa layer and progesterone production via the Δ^4 -ketone pathway.

1.3.6. Steroid activity during the menstrual and oestrus cycle

Seamark, et al., (1974), found that the precise pattern of steroid production by sheep follicles invitro, varied according to the stage of the cycle at which they were removed. For example follicles explanted 12 hours prior to ovulation produced seven times more steroid per unit follicular mass, than follicles explanted 3 days prior to ovulation. At the same time there was a fundamental change both in the kind of steroid produced and in the cell type responsible for steroid synthesis.

Baird and Frazer, (1975), noted that human luteal phase follicles secreted little oestrogen, and that follicles greater than 5 mm in diameter underwent degeneration. McNatty et al., (1975) did not find large active follicles in the ovary of women during the luteal phase.

Kemeter, Salzer, Breitenecker and Friedrich, (1975), have shown that the follicular fluid of the probable preovular human follicle has high levels of oestradiol-17 β and progesterone suggesting activation of the aromatizing and 3 β -hydroxysteroid-dehydrogenase enzyme. The rapid rise in progesterone production appeared to cause a transitory depletion of precursors for androgen and oestrogen production. Edwards, Steptoe, Abraham, Walters, Purdy and Fotherby (1972), aspirated follicular fluid from preovular follicles following parenteral administration of gonadotrophins to women. There was significant linear increase in levels of oestradiol-17 β with increasing levels of progesterone. No correlation between follicular steroid levels and size of follicles was noted, but increasing concentrations of follicular steroids correlated with higher doses of gonadotrophin stimulation. The concentration of progesterone but not oestradiol-17 β was higher in preovulatory follicles than in all follicles combined. These findings tend to substantiate those of Kemeter et al., (1975), insofar that progesterone production is associated with a fall in androgen and oestrogen production.

1.3.7. Cellular localization of steroid activity within the follicle

Channing, (1969), cultured human granulosa, theca and stromal cells separately and found that they secreted different patterns of steroid hormones. The theca tissue was able to secrete 17-hydroxyprogesterone androstenedione and in particular synthesize oestrogen from endogenous precursors like pregnenolone, implicating the theca as the major source of oestrogens. In contrast to Ryan et al., (1966), no interaction between

the theca and granulosa cells in oestrogen synthesis was demonstrated. Granulosa cells secreted progesterone as a major product, however this ability decreased with time with a simultaneous rise in the substrate pregnenolone, suggesting a gradual decline in 3β -hydroxysteroid dehydrogenase activity. The stromal cells were less active steroidogenically, their main contribution being androstenedione and 17-hydroxyprogesterone but no detectable oestrogen.

Oakley and Stitch,(1967), demonstrated that testosterone, rather than androstenedione was the preferred physiological endogenous precursor for oestrogen synthesis in the rat ovary, invitro. Fortune and Armstrong,(1977), have advanced the hypothesis that the preovulatory surge of oestrogen may stem from aromatization by granulosa cells of androgen precursors formed in the cells of rat follicles. Studies with isolated theca and granulosa cell cultures in sheep have also shown that the theca can produce androgen precursors and that the granulosa cells are able to use these precursors to form oestrogen, providing FSH is included in the medium. Furthermore, extravasation of the granulosa cells from the large follicles on days 14-15 of the sheep oestrus cycle, results in a marked reduction of oestrogen production, invivo, clearly indicating that ovarian follicular activity is a result of a combined function of both tissues in this species,(Armstrong,Weiss, Kaethner & Seamark,unpublished). It has been shown that the sheep follicle invitro, releases a wide range of steroids. The application of mass fragmentography to facilitate the dynamic investigations of follicular steroidogenesis has been used. A study by Seamark, Phillipou and McIntosh, (1976), has shown that this approach provides a sensitive, highly specific, yet simple multi-hormone analysis system.

1.3.8. The role of gonadotrophins and cyclic AMP in follicular steroidogenesis

An important development in the investigation of ovarian function was made by Moor, (1973), who showed that the individual follicles explanted from the ovaries of sheep, could be isolated by micro-dissection and maintained separately in organ culture. (His methodology was developed to study the steroid activity of human follicles as illustrated in this thesis). He was able to show that only the largest one or two preovular sheep follicles secreted significant amounts of oestrogen, invitro, without gonadotrophic stimulation. Hay and Moor, (1973), demonstrated that the addition of physiological levels of LH to sheep follicles, invitro, inhibited oestrogen secretion and at the same time induced luteinization of the granulosa cells and the secretion of progesterone. There is now

compelling experimental support for the proposal that the stimulation of ovarian steroidogenesis by LH and FSH is mediated by cAMP (Lindner, Tsafiriri, Leibeirman, Zor, Koch, Bauminger and Barnea, 1974). McIntosh and Moor, (1973), showed that LH stimulated cAMP synthesis in sheep follicles, but that this nucleotide did not in itself have the capacity to induce luteinization and progesterone secretion. Moor, Hay and Seamark, (1975) and Hay and Moor (1975), postulated that following the activation of intracellular cAMP in the theca cells by LH, cellular degeneration of these cells occurs and that cAMP may act as a distant diffusion activator of the granulosa cells. Furthermore they suggested that the mechanism for intercellular transfer of cAMP via the basement membrane may be by way of the complex network of plasma membrane gap junctions. This mechanism would thus protect cAMP from degradation by membrane bound phosphodiesterase. Moor, (1977), cultured sheep theca and granulosa separately in tissue culture and was able to show that the theca was the predominant source of androgen synthesis and that the granulosa cells produced mostly progesterone, which were similar to findings reported by Channing, (1969), for human theca and granulosa cells. He was also able to show that an interaction between theca and granulosa was required for maximal oestrogen production.

Weiss, Seamark, McIntosh and Moor, (1976), demonstrated that cAMP formation predominantly occurred in the thecal compartment of cultured sheep follicles, in response to gonadotrophin stimulation, and that a significant amount of this cAMP was released into the extracellular compartment and appeared to pass into the granulosa cells. This is a new concept, because cAMP has generally been regarded as an intracellular, not an extracellular, mediator of protein hormone action.

Weiss and Armstrong, (1977), were able to show that theca cells from both large and small sheep follicles responded preferentially to HCG than to FSH with respect to cAMP production, whereas granulosa cells from small follicles only responded to FSH. However granulosa cells from large follicles had acquired a thirtyfold capacity to respond to HCG and a threefold decline in their capacity to respond to FSH. Hillensjo, Bauminger and Ahren, (1975) showed that LH induced a transitory rise of androgen and oestrogen secretion followed by inhibition, and a sustained stimulation of progesterone secretion by preovulatory rat follicles

1.3.9. Distribution of collagenous connective tissue in Graafian follicles

Espey, (1976), was able to demonstrate the presence of collagen within the theca layer of mature rat ovarian follicles. Espey and Coons, (1976), isolated two 'collagenolytic' enzymes which were present in rabbit follicles and suggested that they may be active in the digestion of the follicle wall during ovulation.

1.3.10. The role of prostaglandin F in human follicular function

Animal studies have shown the LH causes an increased synthesis of PGF by ovarian follicles both *in vivo* and *in vitro*. In lower animals, there is strong evidence that PGF acts as a mediator of certain LH effects, including maturation and extrusion of the ovum. Plunkett, Moon, Zamecnik and Armstrong, (1975), were able to show that human ovarian follicles, *in vitro*, when treated with gonadotrophins were able to synthesize more PGF than control follicles, and concluded that PGF may play a role in ovulation and may act as a mediator of certain LH functions. This conclusion is supported by Armstrong's work (1974), where he inhibited ovulation in rabbits by intrafollicular injection of indomethacin and prostaglandin F antiserum, however entrapment of the ovum occurred within a normal functional corpus luteum. Ovarian contractility has been shown to increase during the periovular period, following infusions of LH, which may be involved in PGF synthesis which is in turn responsible for ovarian contractility.

1.3.11. Nervous innervation related to the Graafian Follicle

Adrenergic nerve terminals have been demonstrated in the human Graafian follicle, the greatest number being on the side of the follicle closest to the medulla, (Owman, Sjöberg, Svensson and Waller, 1975).

It has been suggested that the autonomic nervous system can affect the mechanical activity of the follicular wall by activating smooth muscle cells within the theca externa, and so alter intrafollicular pressure. This mechanism implicates a neurogenic influence on gonadotrophin controlled follicular development and the ovulation process. On the other hand, Weiner, Wright and Wallach, (1975), have shown that unilateral adrenergic denervation of the rabbit ovary did not alter follicular maturation, ovulation, frequency of conception, ovum pickup or tubal transport. Thus, central neuron efferent mechanisms do not appear to be essential for these ovarian functions. It would appear that the significance and regulation of ovarian contractions and the mechanism of follicular rupture is still unresolved.

1.3.12. Δ^5 -3 β -hydroxysteroid dehydrogenase activity within the Graafian follicle

All the hormonally active steroids are synthesized biologically by pathways involving the oxidation of the Δ^5 -3 β -hydroxyl group to the Δ^4 -3-ketone group. The enzyme system which catalyses this oxidation is Δ^5 -3 β -HSD.

Hay and Moor, (1975), examined the distribution of 3 β -HSD activity in the Graafian follicle of the sheep and related it to oestrogen and progesterone production. They were able to show that high oestrogen secretion by follicles, invitro, was associated with 3 β -HSD activity in the theca interna cells and after the addition of LH to the cultured follicles, oestrogen secretion was inhibited and progesterone secretion enhanced with the appearance of 3 β -HSD activity in the granulosa cells. Friedrich, Breitenecker, Salzer and Holzner, (1974), were able to demonstrate the occurrence of progesterone in the follicular fluid of large preovular human Graafian follicles and histochemical evidence of 3 β -HSD activity in the granulosa cells.

1.4. Embryology of the Human Ovary

The genital organs develop in close association with those of the urinary tract. Both systems arise in the intermediate mesoderm lying on each side of the root of the dorsal mesentery beneath the coelomic epithelium. The genital ridge arises as a visible swelling on the medial aspect of the mesonephros in the 5 week old embryo to become the indifferent gonad. A second structure, the paramesonephric (Mullerian) duct forms as an ingrowth of coelomic epithelium on the lateral aspect of the mesonephric bulge, to eventually become the female genital tract. If the six to seven week foetus does not have the "differentiating" sex or Y chromosome (Scott, 1971), the gonad fails to develop into a testis and therefore develops as an ovary. In other words, the human female state corresponds closely to the neuter, insofar that if there is no "differentiating" sex chromosome and no gonadal development, the individual phenotype will be female. It follows that masculine development requires some positive influence; (1) a functional XY chromosome complement and, (2) gonadal male inductor substances for the full development of the male sexual apparatus. Therefore the first indication that the gonad will develop into an ovary is failure of testicular differentiation of the gonad in the seven week foetus.

For many years there have been conflicting views expressed concerning the origin of the germ cells (Dewhurst, 1971). Their origin is now generally accepted to be in the endoderm before the formation of the mesoderm of the lateral plate and before somite formation (Pinkerton, McKay, Adams and Hertig, 1961). According to Pinkerton, et al, germ cells migrate along the endoderm of the yolk sac into the gut, through the mesenchyme at the root of the mesentery and into the medial aspect of the mesonephros at the site of the primitive gonad. Using histochemical techniques these workers demonstrated germ cells in the 3.7 mm foetus, in the area where the genital ridge would shortly develop. Indeed, it seems probable that the presence of the germ cells is necessary for the subsequent development of the gonad, and that failure to correct migration may predispose to the development of extra gonadal germ cell tumors. Rapid proliferation of germ cells occurs until the 15th week of gestation when maturation occurs and the germ cells become encapsulated by a single layer of primitive granulosa cells to become oocytes. At this stage mitotic division, by which the germ cells have been increasing in number, ceases, and they enter the first stage of meiosis.

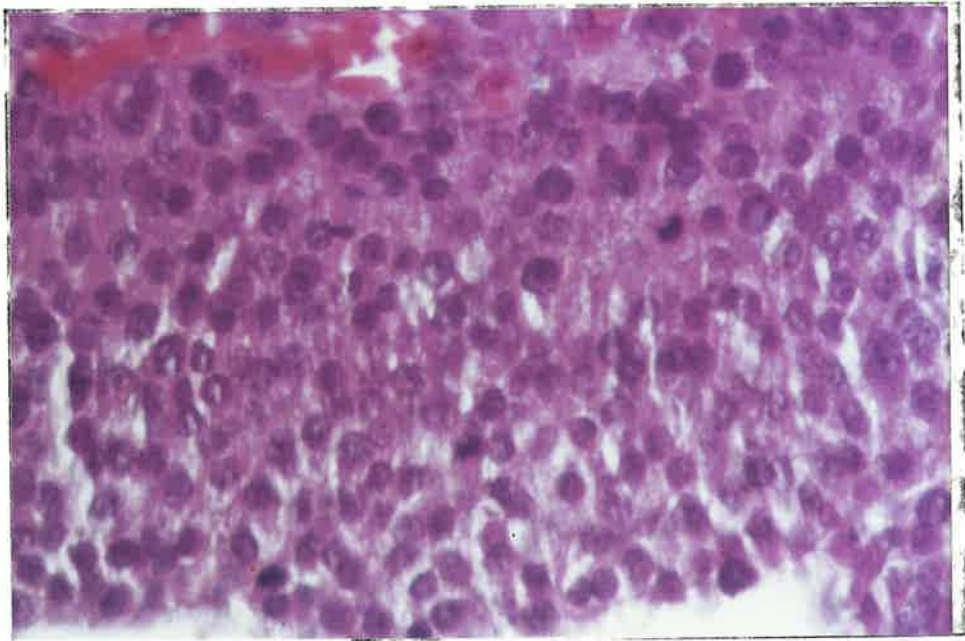
It is thought that the total number of germ cells increases from 600,000 at 2 months gestation to a peak of 7 million at 5 months gestation, but at 40 weeks the number has fallen to 2 million, of which 50% are already atretic. It has been noted that some follicular activity is evident in the ovary at term, (Dewhurst, 1970), the extent of which will not occur again until just before puberty. At the time of the menarche only 400,000-600,000 oocytes are present. It is considered by most authorities that the granulosa and theca cells have a common origin and develop by differentiation from ovarian mesenchyme, (Novak and Woodruff, 1974).

1.5. Histology of the Human Ovary

The ovary is covered by coelomic mesothelium but is usually or almost entirely lacking in the adult ovary. It consists of a single layer of cuboidal epithelium and is well preserved in young children. There is no longer justification for the supposition that in man, germ cells arise from this 'germinal epithelium'. During reproductive life the cortex constitutes two thirds of the ovaries depth and contains a stroma of spindle-celled connective tissue that has many specialized components, ovarian follicles and corpora lutea at all stages of maturation and atresia. A few involuntary muscle fibres are found chiefly around larger blood vessels. The medulla consists mainly of the major ovarian blood vessels, lymphatics and stroma.

1.5.1. The Primordial Follicle

Primordial follicles become fewer in number throughout reproductive life, and are entirely absent after the menopause. The postmenopausal ovary has a much thinner cortex. Apart from primordial follicles, atretic follicles or corpora fibrosa, corpora lutea and albicantia. The primordial follicle consists of a central germ cell or ovum encircled by a flattened or low cuboidal layer of membrane granulosa. As the follicle grows the granulosa becomes definitely cuboidal and later stratified, showing at first two layers and later several layers of cells. Soon the follicle develops a central antrum when it reaches 0.4 mm in diameter, and the ovum is placed at one pole, surrounded by a peninsula like accumulation of granulosa cells, the cumulus oophorus or discus proligerus. The granulosa is devoid of blood vessels and is dependent for its nutrition upon the highly vascular theca interna. The theca externa consists of condensed ovarian stroma which is thought to be inactive steroidogenically.



Photomicrograph 1. (x600)

A section through a 10 mm diameter preovular Graafian follicle. The granulosa layer is 10 cells thick.

1. Three granulosa cells show evidence of mitotic activity.
2. Pavementing of the basal granulosa cells can be seen. The basement membrane is not visible.
3. Blood vessels in outer theca interna.

1.5.2. The Mature Ovarian Follicle

According to Novak and Woodruff (1974), the mature human follicle rarely exceeds 8 mm in diameter. From the observations contained in this thesis, follicles up to 12 mm in diameter have been found which are almost certainly just preovular. Their removal was followed by a fall in blood oestradiol-17 β levels within an hour and withdrawal bleeding within 48 hours. It has been noted that human follicles do not protrude to a marked extent above the surface of the ovary, as is the case in many of the lower animals. Even so it is quite easy to define them visibly at the time of follicle biopsy. The granulosa cells consist of four to ten layers of polyhedral cells, compactly placed, with darkly staining central nuclei with frequent mitoses. Call-Exner bodies are frequent. The antrum contains liquor folliculi. The germ cell area consists of an inner layer of granulosa cells disposed in radial fashion, constituting the corona radiata. Inside is an amorphous birefractile membrane, the zona pellucida. Between this and the ovum is a narrow perivitelline space. The cell wall of the ovum is the vitelline membrane, its nucleus the germinal vesicle, and its nucleolus the germinal spot. Mitotic activity continues in granulosa cells until the 18th day of an ovular 28 day cycle (Novak and Woodruff, 1974). In the present state of our knowledge it is still difficult to test whether a Graafian follicle is in a condition of late normal development or of early atresia. However, the presence of polar bodies in the ovum is said to reflect maturity. According to McKay, Pinkerton, Hertig and Danziger, (1961), where the ovum is normal, follicular structure is histologically similar in follicles from 0.3 to 10 mm in diameter, throughout the menstrual cycle. From a histological point of view, yes, but not from a steroidogenic or histochemical point of view, as will be shown in this thesis.

The theca interna is made up of specialized mesenchymal cells which like the granulosa are highly susceptible to normal and abnormal hormonal influences. They are rich in lipid both before and after ovulation. Prior to ovulation they are separated from the granulosa cells by a distinct basement membrane. The theca interna is very vascular, forming a perigranulosa vascular wreath.

1.5.3. Atresia Folliculi

The vast majority of follicles die long before full maturation, and with each cycle many follicles are thus blighted, but as will be seen in this thesis, they are certainly active steroidogenically prior

to their demise and probably play an important albeit subordinate role to the follicle destined to ovulate, in maintaining reproductive activities. The ovum degenerates first followed by granulosa and theca cells. The cystic stage of atresia is followed soon after by cicatricial obliteration to produce a corpus fibrosum.

1.5.4. Corpus Luteum

The histology of the corpus luteum will not be covered in detail as it is not very relevant to this thesis. Meyer, (1911), was the first to describe in detail the macroscopic and microscopic appearances of the stages from the ruptured follicle to complete involution of the human corpus luteum. He was the first to point out the four recognizable stages of corpus luteum development, namely, proliferation, vascularization, mature or 'blossom stage' and regression. A full account is contained in the work of White, Hertig, Rock and Adams (1951).

CHAPTER TWO

2. STEROID PROFILES OF HUMAN OVARIAN FOLLICLES IN ORGAN CULTURE: CORRELATIONS WITH FOLLICLES REMOVED IN THE EARLY FOLLICULAR, LATE FOLLICULAR, LUTEAL PHASE AND THE IMMEDIATE POST PARTUM PERIOD

2.1. INTRODUCTION

There is now compelling experimental evidence to indicate that in man and many other species the hypothalamic-pituitary system is essentially acyclic and that the ovary itself is the seat of inherent cyclic function (Knobil, 1974; Yen, 1975). Little is known, however, of the intraovarian function which determines this fundamental role. It is recognized that the ovary is comprised of three basic tissues, follicular, consisting of a multitude of follicles at different stages of development, one or more corpora lutea and a stromal matrix. Each tissue functions differently depending upon the phase of the cycle and the hormonal milieu to which it has been, and is being, exposed. Further complexity is introduced when it is recognised that each tissue is a heterogeneous mixture of cell types.

An important development in the investigation of ovarian function was made by Moor, (1973), who showed that the individual sheep follicle could be isolated by micro-dissection and maintained separately in culture. Subsequently, we were able to show that human ovarian follicles could be similarly explanted and maintained in organ culture for periods of up to seven days with good maintenance of steroid release and morphological integrity, (Kerin, Moor and Seamark, 1974 & 1976).

Much of the preliminary experimental work was done using sheep follicles, which were readily available, and physically similar to human follicles. Techniques developed in experiments on sheep could therefore be applied directly to studies of human follicles thus maximising exploitation of this valuable material when it was procurable. I would like to express my sincere thanks to Dr. Robert Moor and Dr. Robert Seamark for giving me their time and knowledge in order to learn the technique for follicle culture.

The experiments reported here are subject to the criticism that can be made against all invitro incubations, particularly with regard to the disruption of the normal relationship between intraovarian compartments, the absent influence of circulating gonadotrophins, and the possibility

of progressive alteration of some of the enzyme systems as the incubation proceeds. Despite these limitations, the studies presented here on isolated human follicles, explanted at defined stages within the reproductive cycle behave differently with respect to steroid activity, *invitro*.

2.2. METHODOLOGY

2.2.1. Criteria for ovarian follicle biopsy

All women included in this study were within the reproductive age group and having spontaneous regular menstrual cycles, which were not modified by exogenous hormonal intake. From March 1974 to August 1977 a total of 87 women consented to ovarian biopsy whilst having operations such as abdominal tubal ligation or hysterectomy.

2.2.2. Ethical Considerations

Initially human ovarian follicles were obtained from whole ovaries which had been removed for medical reasons. Permission to remove follicles was obtained from the surgeon and pathologist involved with the patient's care. Preliminary experiments clearly demonstrated that human ovarian follicles incubated in organ culture, using the techniques of Moor (1973), maintained excellent steroidogenic function and morphological integrity (Kerin, 1974).

In order to obtain sufficient follicles from normal healthy young females it was felt that the ideal subjects would be women undergoing abdominal tubal ligation. An application for permission to perform ovarian follicle biopsies was made to the Research and Ethics Committee of the Queen Elizabeth Hospital, Woodville, South Australia. The application was approved and a consent form designed for the patient to read and then sign to register her written consent (see page). The patient acceptance of this procedure was very good and of all the patient's approached regarding ovarian biopsy there were none who refused consent.

Clinical Details

The name, age and past medical history was documented. In particular, a detailed obstetric and gynaecological history was obtained, the normality of the menstrual cycle, duration and amount of menstrual loss and date of the last menstrual period recorded. Previous contraceptive practice, the ease of conception and ability to maintain a pregnancy was also recorded. It was felt that these clinical details in combination with the steroid and enzyme activity of the explanted follicles in organ culture would give a more complete picture of the patient's *invivo* and *invitro* ovarian function.



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DEPARTMENT OF
OBSTETRICS AND GYNAECOLOG
QUEEN ELIZABETH HOSPITAL
WOODVILLE 5011

JK/TS

27th May, 1975.

Dear Mrs.,

At the present time in this Hospital, we are carrying out research investigations into the function of the human ovary.

For this study we are asking patients who are having an abdominal operation if they would mind if the surgeon removed a very small section of ovary at the time of performing the planned operation.

This extra procedure takes only a few minutes, it will cause no complications nor interfere with the function of your ovaries in any way.

If you are agreeable to the above procedure could you please sign below.

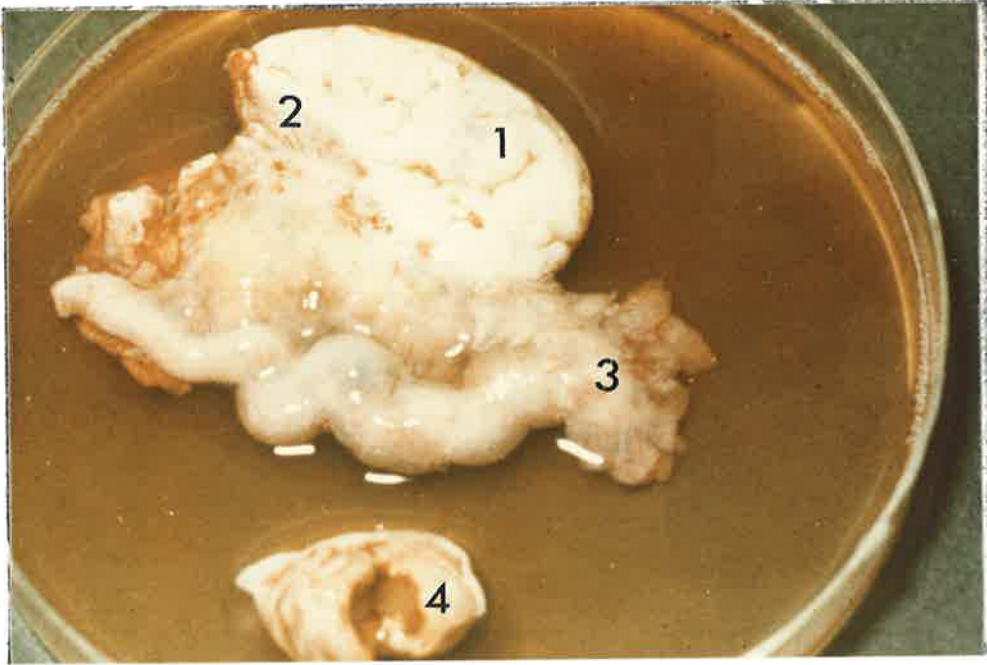
Yours sincerely,

JOHN KERIN, Senior Registrar in
Gynaecology.

SIGNED

DATE

WITNESS



Photograph 2. (x1.5)

Salpingoophorectomy during the luteal phase

1. Small luteal phase follicle (just visible).
2. Site of removal of corpus luteum.
3. Fimbriated end of fallopian tube.
4. Opened corpus luteum.



Photograph 3. (x3)

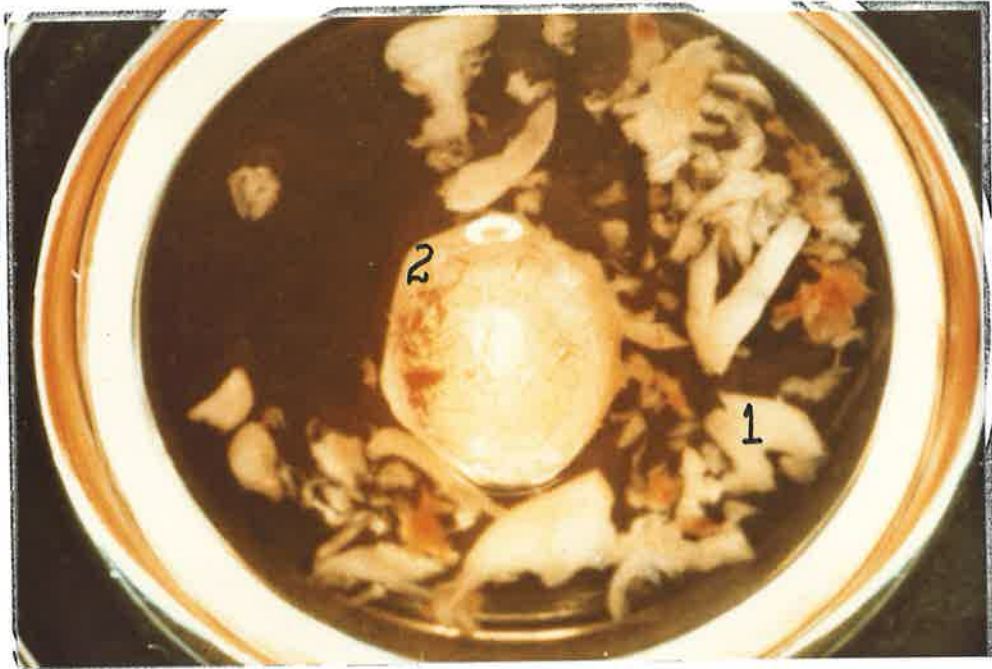
Freshly explanted ovarian follicle encapsulated in ovarian stroma.

Timing of Surgery

In order to obtain a sufficient number of follicles at different stages of the menstrual cycle, surgery was arranged to coincide with the early follicular phase (days 1-7), late follicular phase (days 8-14) and the luteal phase (days 15-28) of a normal 28 day cycle. In this way specific experiments could be designed to assess particular behaviour of certain follicles in relation to their size and hormonal environment at the time of explantation. A series of follicles were also explanted within the first 5 days following parturition at the procedure of post partum tubal ligation. In a number of cases peripheral venous blood was taken prior to surgery, during surgery and following surgery for the estimation of serum prolactin, FSH, LH and oestradiol-17 β .

2.2.3. Surgical technique for ovarian biopsy

In most cases a lower abdominal Pfannestiel incision was made for tubal ligation or hysterectomy. Both ovaries were inspected for normality and the largest follicles identified. If a corpus luteum was present, often a small slice of it was removed as well as surrounding follicles. In all cases the largest visible follicles were explanted from one or both ovaries. The ovary was delivered to the level of the skin incision and a pair of Allis forceps placed on each edge of the ovarian pedicle taking care to avoid the ovarian vessels. A 1-2 centimetre elliptical incision was made through the ovarian cortex around the edge of a follicle. With a pair of very fine single toothed forceps the lip of cortex was elevated and the follicle underneath shelled out using a combination of gentle sharp and blunt dissection. Minimal blood loss occurred during this procedure and haemostasis was always easily achieved by closing the small incision with two or three 2/0 plain catgut interrupted mattress sutures. An atraumatic round bodied needle was always used to carry the suture material. This procedure was repeated up to three times in the one ovary or occasionally also on the other ovary. In a number of cases surrounding follicles of equivalent size to the ones explanted were punctured with a size 25 needle and their follicular fluid aspirated. Estimations of their osmolality, sodium and potassium concentration, prolactin, LH, FSH, testosterone, oestrogen-17 β and progesterone levels were made (values are shown later in the text). After checking that absolute haemostasis had been achieved the abdominal



Photograph 4. (x3)

Course follicle dissection has just been completed and fine dissection about to start.

1. Stromal fragments.
2. Small fragments of attached vascular theca externa.

wall was closed in a routine manner.

An endometrial curette was performed just prior to the ovarian biopsy on 76 of the 86 women who had ovarian biopsies. The histological evaluation of the endometrium was invaluable as a means of dating the endometrium and was a means of checking the validity of the date of the last menstrual period and the stage within the menstrual cycle at the time of explantation.

Post operative sequelae to ovarian biopsy

All patients were seen by the author post operatively. There were no intraoperative or postoperative complications from this small additional procedure. Their stay in hospital and postoperative discomfort was no different from a patient who had undergone an abdominal tubal ligation. Followup of these patients for up to three months postoperatively, indicated that ovarian function as judged by menstrual cyclicity, was not disturbed.

Collection of ovarian follicles

After explantation the follicles were transferred to a sterile container of "Transport Media", (see Materials), which had been kept at a temperature of 4⁰C in a nearby refrigerator within the operating theatre complex. This medium has been found suitable for maintaining ovarian tissue in good condition from the time of surgical removal until dissection. In all cases the facilities necessary for dissection of the ovarian follicles had been prearranged so that there was no delay between explantation and dissection.

2.2.4. Dissection of ovarian follicles

All dissection procedures were carried out with sterile instruments using strict aseptic techniques. When the follicles were exposed to the air for manipulative procedures they were protected from personal and airborne contamination by the use of a Labmaster sterile hood containing an ultraviolet light.

Each follicle was picked out of the transport media using a pair of fine single toothed forceps by grasping a small area of stroma attached to the follicle. The follicle was transferred to a sterile Petri dish, the bottom of which was covered by a shallow layer of "Dissecting Media", (see Materials).



Photograph 5. (actual size)

'Clean' Graafian follicle following fine microdissection.

1. Stainless steel grid.
2. Incubation medium.
3. Falcon dish.

Course Dissection

Using a pair of small curved Mayo scissors and a pair of fine single toothed dissecting forceps the larger fragments of stroma were cut away.

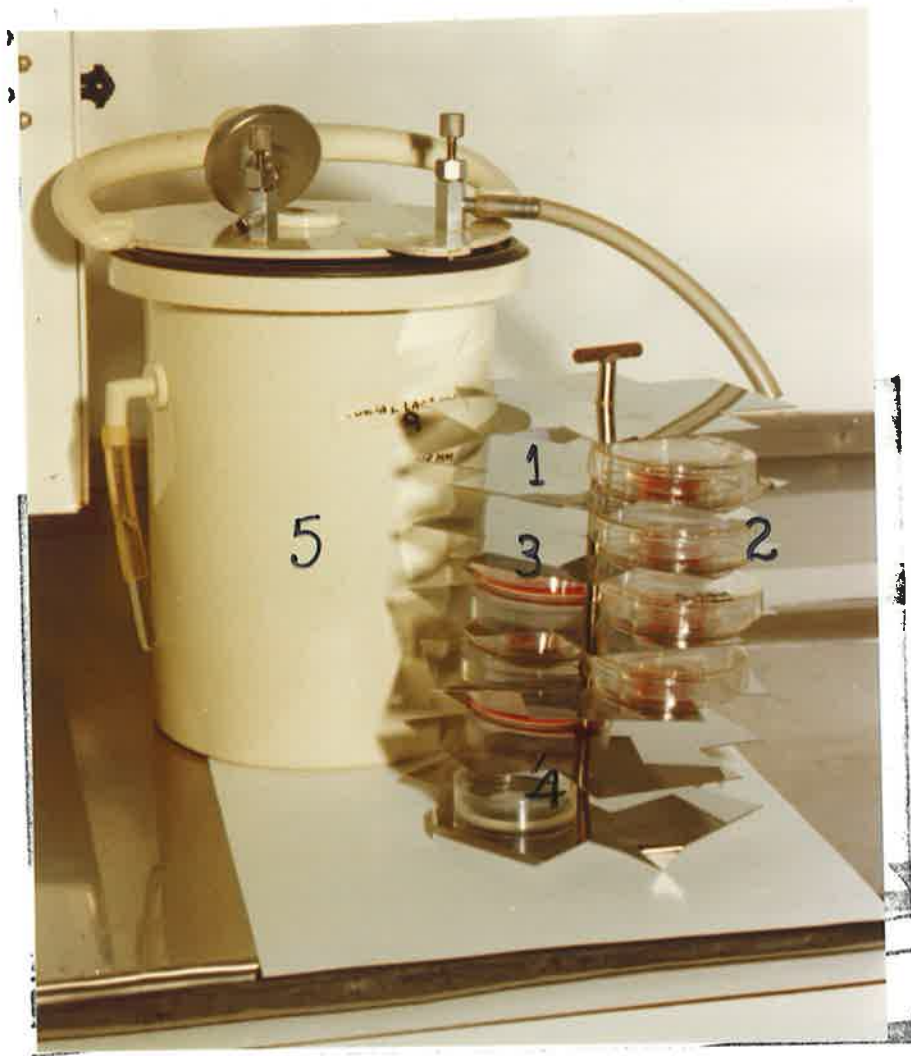
Fine Dissection

With the aid of an Olympus binocular dissecting microscope good visual detail of the remaining fragments of stroma was possible. These fragments were carefully peeled off using two pairs of very fine watchmaker forceps; one being used for traction and the other for counter traction to prevent tearing and rupture of the delicate follicle wall. After the completion of dissection the follicle appeared as a smooth, spherical, translucent, vessel free structure. With the aid of a good light the ovum surrounded by its perigranulosa cells could be made out as a darker shadow within the follicle. While awaiting culture, the Petri dish containing the follicle was flooded with "Dissecting Media" and kept at 4°C.

Preparation for incubation of follicles

Pre sterilized Falcon culture dishes (reference number, 3037), were used to house each follicle. A sterile stainless steel metal grid was placed in the central depression of the dish. This finely meshed grid provided the follicle with an excellent mechanical support and allowed adequate contact between the underside of the follicle and surrounding incubation media. The degree of pressure necrosis from the small areas of follicle wall on the grid was minimal. This was evaluated and found to be so by histological assessment of follicles after incubation.

Incubation media was then added to the Falcon dish taking care to deliberately wet the grid and fill to a level to achieve contact with the undersurface of the grid platform. Each freshly dissected follicle was then transferred to its labelled Falcon dish and placed centrally on the grid using a small bone curette scoop. A final check was made to ensure that sufficient incubation media was in contact with the base of the follicle. The lid of the Falcon dish was then added. This arrangement allowed adequate perfusion of the follicle from the media and adequate exposure to the gas phase which consisted of 45% oxygen (O₂), 5% carbon dioxide (CO₂) and 50% nitrogen (N₂).



Photograph 6.

Incubation apparatus.

1. Culture rack.
2. Right side of rack - Falcon dishes containing whole follicles. Left side Petri dishes containing granulosa monolayers.
4. Open dish of water for humidification.
5. McIntosh & Fieldes anaerobic jar.

It has been noted by Dr. Robert Moor (personal communication, 1974), that a follicle with fragments of stroma still attached has an impairment of both the gas phase and liquid phase perfusion. The diameter of each follicle was recorded using a pair of vernier calipers prior to incubation.

Incubation of follicles

Each Falcon dish containing a follicle was then placed on a stainless steel multilayered support. This support, which is capable of carrying twenty Falcon dishes; is next lowered into a McIntosh and Fields anaerobic jar. An open dish of water was included to provide a humidified atmosphere. After the lid was screwed down to provide an airtight seal, the inlet and outlet valves were opened and the inlet valve connected to a cylinder containing 45% O₂, 5% CO₂ and 50% N₂. A gas flow at the rate of 0.5 litres/minute was maintained for 10 minutes. The outlet valve was closed and the inlet valve also closed 5 seconds later. This procedure had the effect of creating a low pressurized gas phase.

Collection of culture media

At 24 hour intervals the culture media surrounding each follicle was aspirated with a sterile syringe attached to a 23 gauge needle and placed into a preweighed and labelled flat based centrifuge sample tube (Kayline, ref. no. COCS 4016), reweighed and then frozen at -20°C pending steroid analysis. The difference in sample tube weights equaled the weight of culture media aspirated and assuming a mass to density ratio of 1, the steroid levels could be calculated as ng steroid per ml of culture media. An equivalent amount of culture media was introduced and the follicle incubated for a further 24 hours. This procedure was usually performed for 3 to 5 days with each batch of follicles.

Harvesting of Ovarian Follicles

In all cases the follicles themselves were harvested after 3 to 5 days in organ culture and either fixed in formalin for histological evaluation, or frozen at -15°C for histochemical evaluation which was always proceeded with on the same day.

2.2.5. MATERIALS

Transport Media

This consists of Dulbecco Phosphate Buffer, with the addition of Kanamycin Sulphate (5 mg/100 ml of Dulbecco Phosphate Buffer).

Dulbecco Phosphate Buffer is a balanced salt solution, issued by the Commonwealth Serum Laboratories as a sterile, single-strength solution which requires no further dilution. The concentration of each reagent is as recommended by Dulbecco and Vogt (1954). All components have been selected because of their freedom from toxicity to tissues, and the solution is made in deionized distilled water.

Ingredients

Dulbecco Phosphate Buffer contains in every 100 ml.

Sodium Chloride	0.8 g.
Potassium Chloride	0.02 g.
Disodium Phosphate (anhydrous)	0.115 g.
Monopotassium Phosphate	0.02 g.
Calcium Chloride	0.01 g.
Magnesium Chloride	0.01 g.

Dulbecco Phosphate Buffer is recommended for tissue-culture and other procedures in which an isotonic, balanced salt solution is required. Storage at 2°C - 5°C is recommended.

Dissecting Media

This consists of transport media with the addition of 20 ml of calf serum (not inactivated) per 100 ml of transport media.

Incubation Media

This consists of:-

1. Medium 199, single strength solution 100 ml
2. Foetal calf serum (not inactivated) 20 ml

Kanamycin sulphate (powder), 1 mg, plus crystalline beef insulin, 5.6 mg, (26.6 units/mg) is dissolved in 1 ml of N/10 hydrochloric acid, which is then added to the mixture of Medium 199 and foetal calf serum via a millipore filter (13 mm diameter filter with a millipore size of 0.45µ). The concentrated hydrochloric acid is diluted to 1/10 normal strength with Dulbecco Phosphate Buffer.

The ingredients contained in Medium 199 are set out as listed in Cell Culture Handbook (1974) issued by the Commonwealth Serum Laboratories, catalogue No. DM01., page A2.2.

Medium 199 (Dried Powder)

Ingredients per 1000 ml equivalent:

NaCl	8.0	g	Guanine	0.3	mg
KCl	0.4	g	Xanthine	0.3	mg
MgSO ₄ .7H ₂ O	0.2	g	Hypoxanthine	0.3	mg
Na ₂ HPO ₄	0.06	g	Thymine	0.3	mg
KH ₂ PO ₄	0.06	g	Uracil	0.3	mg
CaCl ₂	0.14	g	Pyridoxal. HCl	0.025	mg
Fe(NO ₃) ₃ .9H ₂ O	0.1	mg	Pyridoxine. HCl	0.025	mg
Glucose	1.0	g	Niacin	0.025	mg
Adenine Sulphate	0.01	g	Niacinamide	0.025	mg
l-Arginine. HCl	0.07	g	p-Aminobenzoic Acid	0.05	mg
l-Histidine. HCl	0.02	g	i-Inositol (meso)	0.05	mg
l-Lysine. HCl	0.07	g	Riboflavin	0.01	mg
dl-Tryptophane	0.02	g	Thiamin. HCl	0.01	mg
l-Tyrosine	0.05	g	Calcium Pantothenate	0.01	mg
l-Cystine	0.02	g	Choline Chloride	0.5	mg
dl-Phenylalanine	0.05	g	D-Biotin	0.01	mg
dl-Methionine	0.03	g	Folic Acid	0.01	mg
dl-Serine	0.05	g	Calciferol	0.1	mg
dl-Threonine	0.06	g	Cholesterol	0.2	mg
dl-Leucine	0.12	g	Alpha-Tocopherol	0.01	mg
dl-Isoleucine	0.04	g	Mendione	0.01	mg
dl-Valine	0.05	g	Ribose	0.5	mg
dl-Glutamic Acid. H ₂ O	0.15	g	Desoxyribose	0.5	mg
dl-Aspartic Acid	0.06	g	Adenylic Acid	0.2	mg
dl-alpha Alanine	0.05	g	Phenol Red	0.02	g
l-Proline	0.04	g	l-Cysteine. HCl	0.1	mg
l-Hydroxyproline	0.01	g	Glutathione	0.05	mg
Glycine	0.05	g	Ascorbic Acid	0.05	mg
l-Glutamine	0.1	g	Vitamin A Acetate	0.1	mg
CH ₃ COONa. 3H ₂ O	83	mg			
Adenosine Triphosphate	10.9	mg			

MATERIALS

Particular surgical, dissecting and incubation equipment

1. Two pairs of Babcocks or Alice forceps.
2. One pair of Adson toothed forceps.
3. One pair of Adson-Brown (with atraumatic teeth) forceps.
4. One No. 3 scoped handle and a size 15 blade.
5. One pair of curved dissecting scissors ($4\frac{1}{2}$ " strabismus type).
6. One pair of Metzenbaum (or McIndoe) $5\frac{1}{2}$ " curved scissors.
7. 2/0 chromic catgut on an atraumatic needle (G 123).
8. Two pairs of watchmaker forceps (No. 4, Hipil or Inox).
9. Small bone curette.
10. Prepacked sterile transport pots of Petri dishes.
11. Stainless steel metal grids (1 x 1 cm sq. tops with 0.7 mm grid spaces).
12. Olympus binocular dissecting microscope (magnification used, 10 x 0.7).
13. Labmaster sterile hood.
14. Falcon culture dishes (50 mm diameter, 13 mm deep, ref. no. 3037).
15. Cylinder containing 5% CO₂, 45% O₂ and 50% N₂.
16. McIntosh and Fields anaerobic jars.
17. Qualtex Incubator (water jacketed, thermostatically controlled).
18. Stainless steel multi layered rack for Falcon dishes.
19. Disposable plastic syringes (5 ml) and size 25 and 21 gauge needles.
20. Flat based centrifuge sample tubes (Kayline, ref. no. COCS 4016).
21. Gas sterilization equipment for plastic apparatus and Millipore filters.
22. Dry heat sterilization equipment for glassware and surgical instruments.

2.2. METHODOLOGY

2.2.6. Radioimmunoassay procedures for testosterone, oestrogen and progesterone

The radioimmunoassay procedures used in this series of experiments were developed for the specific antisera used against testosterone, oestradiol-17 β and progesterone by Meg Kaethner, Professional Officer (Class I), Department of Obstetrics & Gynaecology, Queen Elizabeth Hospital, Adelaide. Assays for testosterone, oestrogen and progesterone were carried out under the supervision of M. Kaethner.

The antiserum to testosterone -3-(0-carboxymethyl) oxime conjugated to bovine serum albumin was donated by Drs. R.I. Cox and M. Wong, CSIRO, Blacktown, N.S.W. Australia. The evaluation of antisera specificity against both oestradiol-17 β and progesterone was kindly performed by M. Kaethner.

2.2.7. Measurement of specificity of the antisera

Specificity of the oestradiol-17 β and progesterone antisera were determined using the radioimmunoassay procedure outlined on page Several concentrations of those steroids expected to be present in the media in significant amounts were reacted with a fixed dilution of antibody (selected to produce 60-70% binding of the tritiated steroid), thus enabling comparison of the inhibition curve of the test steroid with the inhibition curve of the steroid to be assayed.

All incubations were carried out in phosphate buffered 0.9% saline (pH 7.0, 0.1M), containing 0.1% bovine serum albumin and 0.1% sodium azide. The tritiated steroid was made up in this buffer so as to contain 10,000 cpm per 0.1 ml for oestradiol-17 β and 20,000 cpm per 0.1 ml for progesterone. Standard solutions of the test steroids were made up in absolute ethanol and differing volumes of these aliquotted in triplicate into tubes, resulting in masses ranging from 0.1 ng to 1000 ng. Normal standard curves for progesterone and oestradiol-17 β were set up in parallel. After evaporation of the ethanol with air, 0.1 ml of the respective tritiated steroid was added to all tubes which were then equilibrated for 30 minutes at 37 $^{\circ}$ C to solubilize the non-radioactive steroid. The antibody was then added in 0.1 ml phosphate buffer to result in the dilution used in the normal assay procedure (progesterone, 1:2000; oestradiol-17 β , 1:16000).

After 5 minutes at 37°C, all tubes were vortexed briefly and placed in an ice-bath.

Separation of free and antibody-bound fractions was achieved by precipitation with polyethylene glycol after addition of carrier gamma-globulin as described in the radioimmunoassay procedure (page The progesterone standards and test steroids were equilibrated for 30 minutes on ice before precipitation and oestradiol-17 β for one hour before precipitation. Two ml of toluene-ethanol scintillant was added to the precipitate after aspiration of the supernatant and the radioactivity in the bound fraction determined in a liquid scintillation spectrometer.

The results were plotted (figure 1) as the percentage of tritiated oestradiol-17 β or progesterone bound at a certain mass of the test steroid as compared to that bound with no added steroid (% Bx/Bo) against log of the mass of steroid added. The percentage cross reaction of, say, steroids with oestradiol-17 β has been defined by Abraham (1969) as:-

mass of oestradiol-17 β required to displace 50% of the oestradiol-17 β -H ³ bound to the antiserum	/	mass of steroid required to displace 50% of oestradiol-17 β -H ³ bound to the antiserum
---	---	--

Table 1 (page 38) shows the respective values for the steroids tested against the progesterone and oestradiol-17 β antisera together with values sent with the testosterone antiserum by courtesy of Drs. R.I. Cox and M. Wong.

Steroid assay procedures

Testosterone, progesterone and oestrogen were all assayed directly from the culture media. Each steroid was assayed by a radioimmunoassay procedure using polyethyleneglycol 6000 for precipitation of the bound steroid. The testosterone antiserum was used at a dilution of 1:20,000. The oestrogen antiserum was raised against oestradiol-17 β , 6-(O-Carboxymethyl) oxime conjugated to bovine serum albumin (BSA) and used in a dilution of 1:16,000. The progesterone antiserum was raised against progesterone conjugated through the 11 position to BSA and used at a dilution of 1:2000.

FIGURE 1

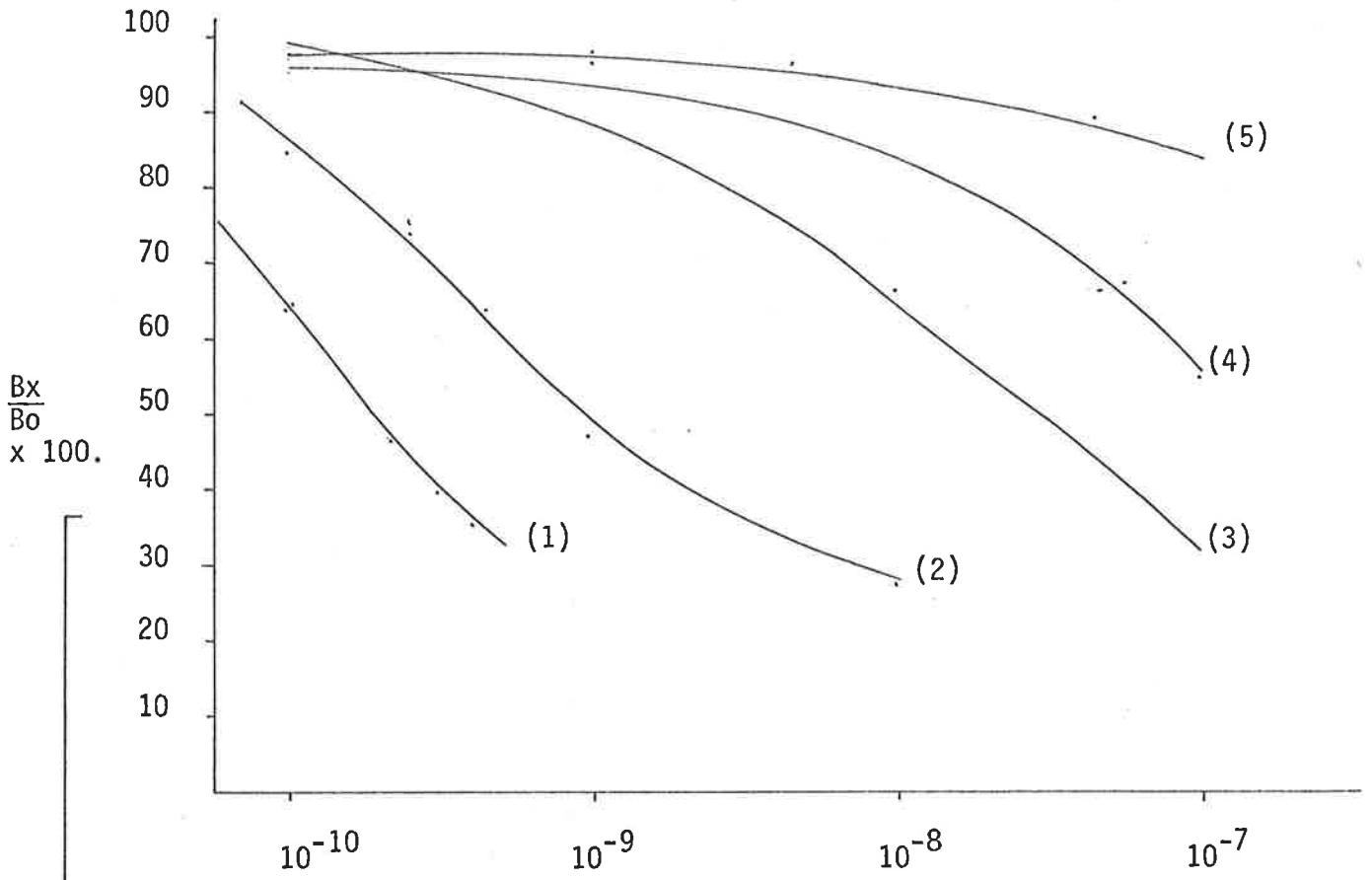
Graph 1. Representation of antisera specificity for

(1) <u>oestradiol-17β</u>	100%
and cross reaction with:-	
(2) oestrone	17%
(3) oestriol	0.6%
(4) testosterone	0.2%
(5) progesterone	< 0.1%

Graph 2. Representation of antisera specificity for

(1) <u>progesterone</u>	100%
and cross reaction with:-	
(2) 5 α -pregnandione	6%
(3) 20 α -OH-progesterone	1%
(4) 17 α -OH-progesterone	0.2%
(5) testosterone	0.6%
(6) Δ 5-pregnenolone	< 0.1%
(7) 5 α -pregnanediol	< 0.1%
(8) oestradiol-17 β	< 0.1%

Graph 1



Graph 2

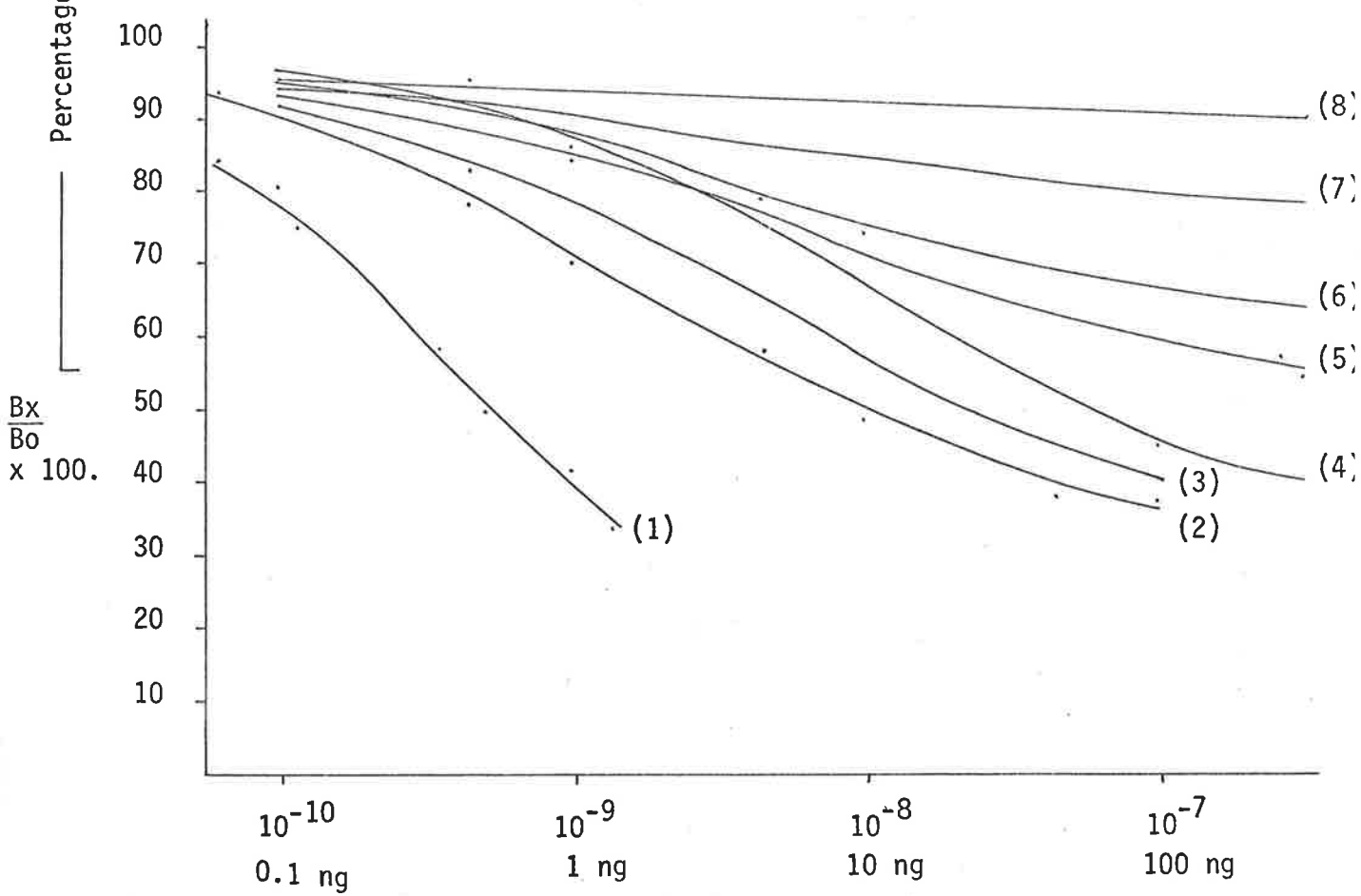


Table 1.

<u>COMPOUND TESTED</u>	<u>PERCENTAGE CROSS REACTION</u>		
	anti- Prog-11-CMO-BSA	anti- E ₂ -17β-6-CMO-BSA	anti- Testosterone-3-CMO-BSA
Oestradiol-17β	< 0.1	100	0.1
Progesterone (4-Pregnene-3,20-dione)	100	< 0.1	0.004
Testosterone (17β-Hydroxy-4-androsten-3-one)	0.6	0.2	100
Oestrone	-	17	< 0.003
Oestriol	-	0.6	< 0.003
17α-Hydroxyprogesterone	0.2	-	< 0.003
20α-Hydroxyprogesterone	1	-	-
5α-Pregnane-3,20-dione	5	-	-
Androstenedione	-	-	1.3
5α-Dihydrotestosterone	-	-	31

METHODOLOGY2.2.8. Radioimmunoassay for OESTRADIOL-17 β

1. Preparation

SolutionsA. Phosphate Buffer : 0.1M, pH 7.0

Make up one litre with and one without 1 g of Bovine Serum Albumin

6.1 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

10.9 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

or

24.6 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

or

8.6 g Na_2HPO_4 (anhydrous)

9.0 g NaCl

1.0 g NaN_3

Make up to 1000 ml with distilled water. Adjust pH 7.0 with concentrated NaOH (may be stored at 4°C for 2-3 weeks).

B. Antibody

1. Use 0.1 ml of a 1:8000 dilution of antibody.

2. 1 mg of the freeze dried antibody up to 40 ml with buffer results in a 1:8000 dilution.

3. Therefore, 2 mg of antibody dissolved in 0.5 ml phosphate buffer containing BSA gives 1 : 50 stock solution (may be stored at 4°C for 2-3 weeks).

4. Working dilution 1:8000.

0.1 ml of 1 : 50 stock + 16 ml phosphate buffer (+BSA)

0.05 ml of 1 : 50 stock + 8 ml phosphate buffer (+BSA)

C. Standard Oestradiol (E_2) Solutions

A. 0.2 mg E_2 per ml EtOH

B. 500 ng/ml

0.25 ml Standard A made up to 100 ml with EtOH

C. 10 ng/ml

0.2 ml of B made up to 10 ml with EtOH (made up fresh every 2-3 days to avoid errors due to evaporation of EtOH).

Aliquots for standard curve

pg E ₂	μl C Standard
25	2.5
50	5.0
100	10
200	20
300	30
400	40
500	50

D. Tritiated E₂ (³H-E₂) Incubation Buffer

- 60 μl ³H-E₂ (2,4,6,7 (n) ³H-Oestradiol in Benzene EtOH)
50 ml Phosphate buffer (+ BSA)
Made up to give 10000 cpm/0.1 ml. Lasts 2-3 weeks.
- Use 0.1 ml of this incubation buffer per assay tube.

E. Gamma Globulin

- Use 50 μl (1 mg) of gamma globulin per assay tube.
- 0.5 g gamma globulin made up to 25 ml with phosphate buffer (may be stored at 4°C for 2 weeks).
- Make up very carefully. Add the gamma globulin to the surface of 20 ml buffer and stir with a magnetic stirrer, allowing it to dissolve slowly. If shaken it will foam and the volume will then be difficult to determine.

F. Polyethylene Glycol 6000 : 27 per cent

- 0.8 ml per tube results in a final concentration of 20.6% of polyethylene glycol.
- 27 g polyethylene glycol 6000 (preferably BDH) made up to 100 ml with phosphate buffer (minus BSA).
- Warm in a beaker of hot water to dissolve. Cool to room temperature before making up to final volume.

G. Scintillant

700 ml Toluene Scintillant
plus 300 ml Redistilled ethanol

II. Procedure

A. Standards

1. Add 0.1 ml EtOH to each standard tube.
2. Aliquot standards in triplicate through a concentration range 25 pg to 500 pg. Expel the standard directly into the solvent in the bottom of the tube. Remember to carefully wipe the needle of the SGE before dispensing each aliquot.
3. Take to dryness under air or N₂.

B. Assay for Oestradiol-17 β

1. Add 0.1 ml ³H-E₂ incubation buffer to all tubes using Hamilton Repeating dispenser. Add 0.1 ml to 4 tubes labelled 'total', add 2 ml scintillant to these and cap.
2. Dispense directly into the incubation buffer, volumes of the culture medium resulting in a mass ranging from 50 to 400 pg of oestradiol-17 β . Take care to wipe the needle of the syringe carefully between aliquots and rinse syringe in distilled water between samples.
3. Equilibrate 10-15 minutes, 37^oC.
4. Add 0.1 ml E₂ antibody (1:8000 dilution) to all tubes using Hamilton Repeating dispenser. Equilibrate 5 minutes, 37^oC.
5. Place in ice-bath and vortex (2-3 seconds). Equilibrate for 1 hour in an ice-bath.
6. Separate with PEG 6000.
 - (a) Add 50 μ l (1 mg) gamma globulin to every tube.
 - (b) Add 0.8 ml 27% PEG 6000 in phosphate buffer to every tube. Vortex vigorously.
 - (c) Centrifuge 20-25 minutes at maximum rpm.
 - (d) Aspirate supernatant with vacuum pump.
 - (e) Add 2 ml scintillant per tube, cap and vortex briefly.
7. Count in Isocap : time : 10 minutes.

Counts : 2000 (5% counting error)
8. Computer programme : RIAH
Instruction file : RIAINSMEG₃

Radioimmunoassay for Testosterone

The basic methodology is the same as outlined for oestradiol-17 β . The differences will therefore only be mentioned.

I. Preparation

A. Phosphate Buffer (unchanged)

B. Antiserum

Working antiserum dilution 1:20,000.

Use 0.02 ml of 1:25 stock + 8 ml phosphate buffer (+ BSA)

0.04 ml of 1:25 stock + 16 ml phosphate buffer (+BSA)

Mix gently and made up fresh each day.

C. Standard testosterone (T) solutions (unchanged)

Non specific binding (NSB) - use 0.5 ml of B standard.

D. Tritiated Testosterone (³H-T) incubation buffer

1. 60 μ l ³H-Testosterone.

50 ml phosphate buffer (+BSA)

Made up to give 8000 cpm/0.1 ml. Lasts 2-3 weeks.

2. Use 0.1 ml ³H-Testosterone incubation buffer per assay tube.

E. Gamma Globulin (unchanged)

F. Polyethylene Glycol 6000:27% (unchanged)

G. Scintillant (unchanged)

Use 2 ml per assay tube.

II. Procedure

A. Standards (unchanged)

B. Assay for testosterone

After the addition of the culture media to incubation buffer allow to equilibrate for 30 minutes at 37^oC. After the addition of the standard antiserum (1:20,000) allow to equilibrate in an ice-bath for ½ hour then separate antibody bound and free hormone with PEG 6000, as described for oestradiol-17 β .

Radioimmunoassay for Progesterone

The following differences from the oestradiol-17 β assay are listed.

1. Working antiserum dilution 1:2000.

2. The incubation times are the same as those outlined for the testosterone assay.

2.2.9. Sensitivity

The limit of assay sensitivity for oestradiol-17 β and testosterone was 0.025 ng per assay tube and for progesterone 0.05 ng.

Assay of blank media for steroids

Blank media was included in each assay for detection of steroids and on all occasions, registered levels were less than 0.020 ng per assay tube. The blank values were not subtracted from culture media values of steroid as their levels were below levels of significance.

2.2.10. Assessment of statistical significance

Comparisons between means were made using the Student's t-test; statistical significance was determined at the 0.1% level. In most cases a single-tailed test could be used, because expected results were either greater or less than the control values. In the situation where the observed value could have been greater or less than the control value a two-tailed test was used to ascertain the level of significance.

Statistical analysis of the data included the determination of the mean value, the standard deviation, the standard error of the mean (SEM), the variance (v) and the number of values in the sample. The mean value \pm SEM are included in the text and the standard deviation (SD) and variance are included along with the mean and SEM in the appendix.

2.2.11. Validation for relating human follicular diameter to follicular weight

The initial work to determine a relationship between follicular diameter and its weight was carried out on sheep follicles by Seamark, Moor & McIntosh (1974). These relationships were determined by weighing a series of 95 follicles varying from 2 to 8 mm in diameter, after removing all stroma by microdissection and aspiration of follicular fluid.

If one assumes a tissue density of 1, then mass equals volume. By plotting a graph of follicular diameter against wet weight a means of estimating a follicle's weight after recording its diameter is easily obtained. It was not possible to sacrifice enough human follicles in this way to see if the same quantitative relationship held. This was because the work involved in obtaining human follicles was considerable and it was felt more valuable to study their steroid activity in organ culture as intact follicles. Following organ culture all follicles were processed either histologically or histochemically. By means of an eyepiece micrometer (1 division = 5μ at 125 x magnification), it was possible to accurately measure follicular wall thickness (5 measurements per follicle were taken and the average thickness recorded). At the same time the relative thickness of the theca interna and membrana granulosa could be determined.

2.2.12. Histochemical assessment of follicle wall thickness

It was found that the process of frozen section and incubation for histochemical purposes tended to disperse the granulosa cell layer particularly and therefore a falsely increased wall thickness determined and a ratio of theca to granulosa wall thickness falsely in favour of the granulosa layer. This method was therefore abandoned.

Histological assessment of follicle wall thickness

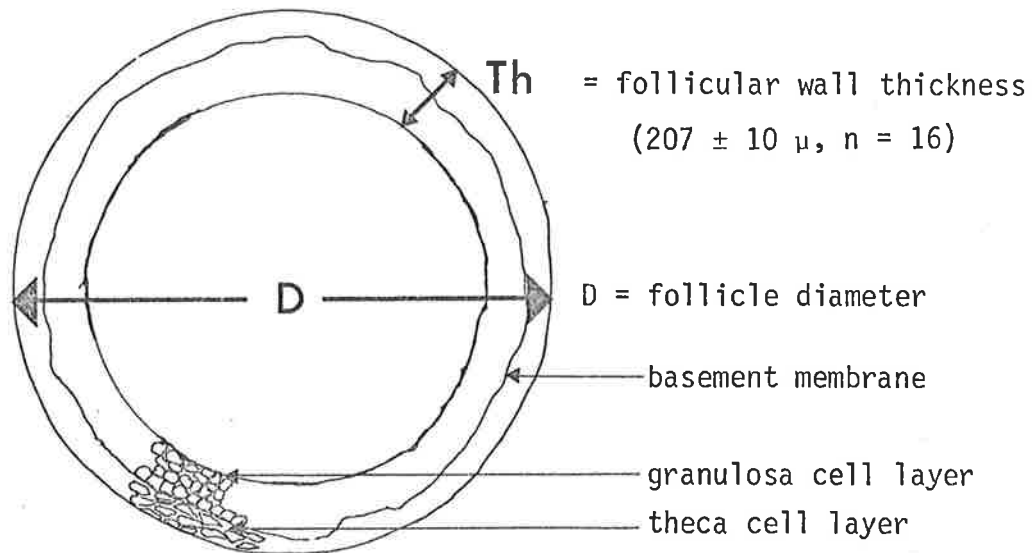
After fixation of ovarian follicles in buffered formalin and staining with haematoxylin and eosin, good anatomical preservation of the theca and granulosa layers was maintained. It was felt that the total wall thickness, theca layer and granulosa layer thickness were truly representative. According to Culling (1974) a correction factor due to tissue shrinkage from the histological processing must be taken into account.

Fixation of the follicles in formaldehyde produces tissue swelling in the order of 20%. The addition of ethanol causes dehydration with resulting tissue shrinkage in the order of 30% and infiltration of the tissue with paraffin wax causes further shrinkage in the order of 30%. From the data presented, it appears that the final result of formalin fixation and paraffin processing is a total shrinkage in the order of 30-40%. A correction factor of 1.35 was therefore used to account for shrinkage in the estimation of follicle wall thickness.

Mathematical calculation of follicular tissue volume after recording follicular diameter

Density = Mass/Volume, assuming a density of 1, mass = volume

$$\text{Volume} = 4/3 \pi \text{ radius}^3$$



$$\text{Total follicle volume } (V_1) = 4/3 \pi (D/2)^3$$

$$\text{Volume of follicular cavity } (V_2) = 4/3 \pi (D-Th/2)^3$$

$$\begin{aligned} \text{Therefore volume of follicular wall} &= 4/3 \pi (D/2)^3 - 4/3 \pi (D-Th/2)^3 \\ &= 4/3 \pi [(D/2)^3 - (D-Th/2)^3] \\ &= 0.52359 (D^3 - (D-Th)^3) \end{aligned}$$

Average follicular wall thickness for follicles ranging from 3-12 mm in diameter = 207 μ ± 10 (SEM, n = 16). This follicular wall thickness of human follicles compares closely with that derived for sheep follicles calculated from weight regression analysis, where the average follicular wall thickness for follicles ranging from 3-8 mm was 209 μ ± 5 (n = 95), Seamark et al (1974).

$$\text{Therefore follicular wall volume} = 0.52359 (D^3 - (D-207)^3)$$

As there was no significant difference between follicular wall thickness of sheep and human ovarian follicles ranging in diameter from 3-8 mm the calculations performed by Seamark et al (1974) were used.

Human follicles with diameters from 8-12 mm were calculated as follows:-

$$\begin{aligned} \text{Follicle Diameter} &= 9 \text{ mm} \\ Y &= \text{mass of follicular tissue} \\ \text{therefore } Y &= 0.524 (9^3 - (9-0.207)^3) \\ Y &= 24.96 \text{ mg.} \end{aligned}$$

$$\text{Follicle Diameter} = 10 \text{ mm, } Y = 30.86 \text{ mg.}$$

$$\text{Follicle Diameter} = 11 \text{ mm, } Y = 39.75 \text{ mg.}$$

$$\text{Follicle Diameter} = 12 \text{ mm, } Y = 46.55 \text{ mg.}$$

Ratio of theca to granulosa wall thickness

$$\text{Theca/Granulosa} = 88/119 = 0.74$$

Therefore the weights of separated theca and granulosa tissue were determined from the relationship:-

$$\text{Theca layer mass (mg)} = \frac{\text{Theca layer thickness}}{\text{Follicle wall thickness}} \times Y \text{ (follicular mass)}$$

$$\text{e.g. for a follicle of 5 mm diameter, } Y = 7.89 \text{ mg.}$$

$$\text{Therefore Theca mass} = 88/207 \times 7.89/1 = 3.35 \text{ mg.}$$

$$\text{Therefore Granulosa mass} = 7.89 - 3.35 = 4.54 \text{ mg.}$$

Although follicular weight in relation to diameter is comparable in the human and sheep for follicles ranging from 3-8 mm diameter, the relative weight of the theca to granulosa does not appear to follow. In fact the ratio of theca to granulosa wall thickness in the human follicle compared to the sheep follicle is as follows:-

$$\text{Human theca/granulosa} = 88/119 = 0.74$$

$$\text{Sheep theca/granulosa} = 55/110 = 0.5$$

Calculation of steroid released (ng) per mg of follicular tissue/24 hours

$$\frac{\text{ng of steroid/mg of follicular tissue per 24 hours} = \text{ng steroid per ml of culture media} \times \text{Volume of media}}{\text{Follicular tissue mass (mg)}} / 24 \text{ hours}$$

Follicular tissue mass (mg)

Although a large follicle may produce a considerable amount of steroid, it must be appreciated that this formula for estimating steroid release per unit mass of follicle, reduces this figure by a much larger degree when compared to a small follicle; e.g. the denominator for a 12 mm follicle is 46.55 whereas for a 4 mm follicle is 4.99.

CALCULATION OF FOLLICULAR DIMENSIONS

(Correction factor x 1.35 for tissue shrinkage)

Follicular Diameter (mm)	Total Wall Thickness (μ)	Theca Layer Thickness (μ)	Granulosa Layer Thickness (μ)	Granulosa Cell Size (μ)	Theca Cell Size (μ)
2	80	40	40	15	14
2	100	60	40	14	12
3	210	100	110	15	16
3	180	90	90	16	17
3	150	70	80	16	17
4	150	60	90	17	18
4	150	70	80	16	16
4	200	90	110	16	18
5	210	90	120	17	18
6	220	100	120	18	17
7	210	80	130	18	18
7	200	80	120	17	18
8	220	90	130	16	17
8	230	100	130	17	17
9	220	100	120	16	17
10	230	90	140	16	16
11	240	110	130	15	16
12	300	80	220	16	17
<u>Statistical analysis</u>					
\bar{X}	207	88	119	16.4	17.1
v	1460	180	1066	0.8	0.6
SD	38	13	33	0.9	0.8
SEM	10	3	8	0.2	0.2
n	16	16	16	16	16

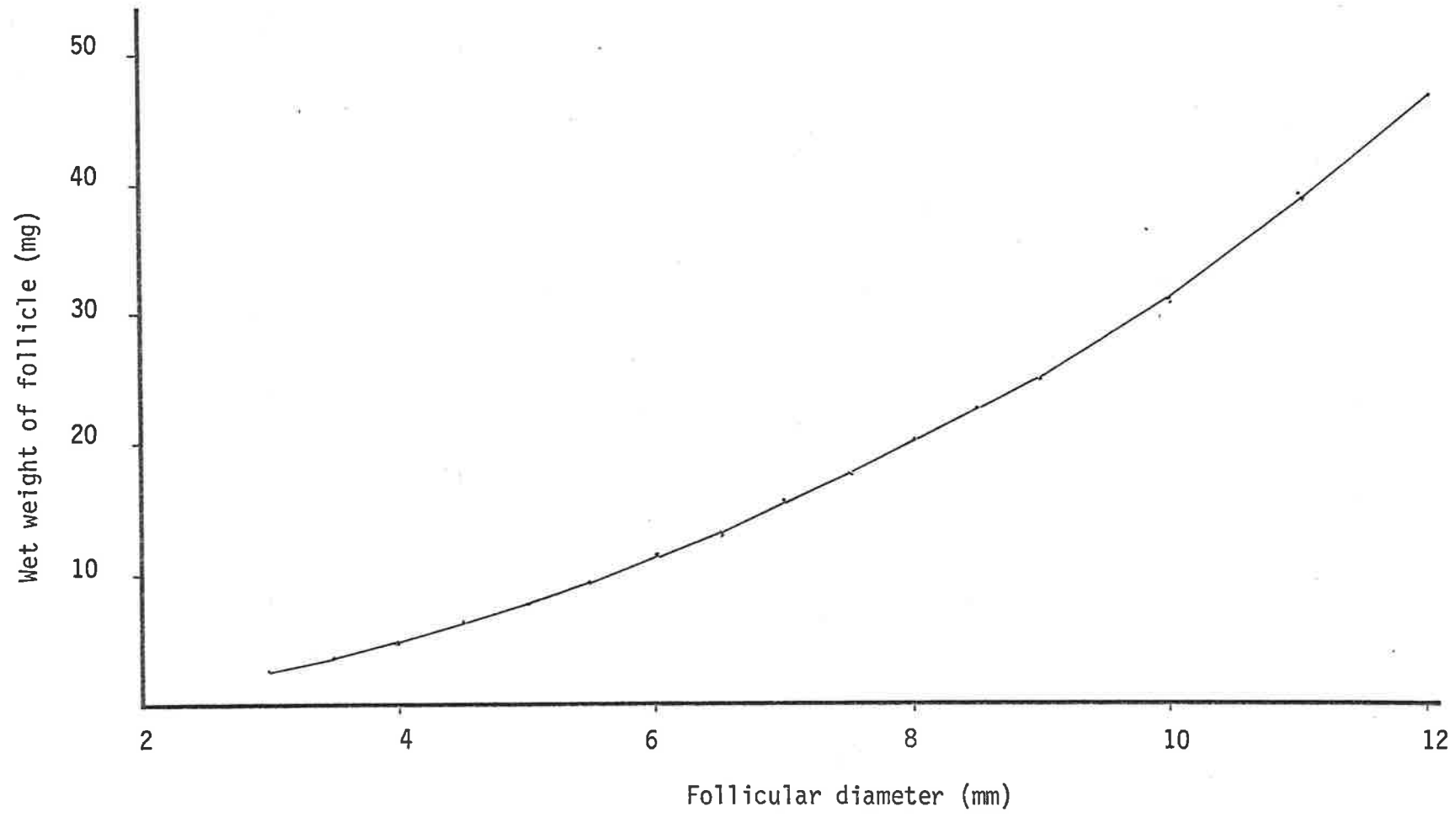
CORRELATION BETWEEN DIAMETER AND WET WEIGHT OF FOLLICLES
(wet weight = theca and granulosa minus follicular fluid)

Relation $Y = 0.523 D \exp 3 - (D - 0.21) \exp 3$
 $D = \text{diameter (mm)}$
 $Y = \text{wet weight (mgs)}$

D	Y	THECA	GRANULOSA
2.0	1.18	0.42	0.76
2.1	1.31	0.47	0.84
2.2	1.44	0.51	0.93
2.3	1.58	0.56	1.02
2.4	1.73	0.61	1.12
2.5	1.89	0.67	1.21
2.6	2.05	0.73	1.31
2.7	2.22	0.79	1.42
2.8	2.39	0.85	1.54
2.9	2.57	0.92	1.65
3.0	2.76	0.98	1.76
3.1	2.95	1.05	1.90
3.2	3.15	1.12	2.03
3.3	3.36	1.2	2.16
3.4	3.57	1.27	2.30
3.5	3.79	1.35	2.44
3.6	4.02	1.43	2.59
3.7	4.25	1.52	2.73
3.8	4.49	1.60	2.89
3.9	4.74	1.69	3.05
4.0	4.99	1.78	3.21
4.1	5.25	1.87	3.32
4.2	5.52	1.97	3.55
4.3	5.79	2.06	3.73
4.5	6.36	2.27	4.08
4.6	6.65	2.37	4.28
4.7	6.95	2.48	4.47
4.8	7.25	2.59	4.66
4.9	7.57	2.70	4.86
5.0	7.89	2.81	5.07

D	Y	THECA	GRANULOSA
5.1	8.21	2.93	5.28
5.2	8.54	3.05	5.49
5.3	8.88	3.17	5.71
5.4	9.22	3.29	5.93
5.5	9.59	3.42	6.15
5.6	9.94	3.55	6.38
5.7	10.30	3.68	6.62
5.8	10.67	3.81	6.86
5.9	11.05	3.94	7.11
6.0	11.44	4.08	7.35
6.10	11.83	4.22	7.60
6.2	12.22	4.36	7.86
6.3	12.63	4.51	8.11
6.4	13.04	4.65	8.38
6.5	13.46	4.80	8.66
6.6	13.88	4.95	8.93
6.7	14.31	5.11	9.20
6.8	14.75	5.26	9.48
6.9	15.19	5.42	9.77
7.0	15.64	5.58	10.06
7.1	16.10	5.75	10.35
7.2	16.56	5.91	10.65
7.3	17.03	6.08	10.95
7.4	17.51	6.25	11.26
7.5	17.99	6.42	11.57
7.6	18.48	6.60	11.88
7.7	18.98	6.78	12.20
7.8	19.48	6.96	12.52
7.9	19.99	7.14	12.85
8.0	20.51	7.32	13.19
* 9	24.96		
10	30.86		
11	39.75		
12	46.55		

Regression of wet weight on diameter



2.2.13. Histological dating of the endometrium

Seventy six patients in this study had endometrial dating from curettings sampled at the time of ovarian follicle biopsy. This histological assessment was performed by the author and checked by Dr. James Kirkland, Director of Cytology and Gynaecological Pathology, Queen Elizabeth Hospital.

The criteria used were described by Novak and Woodruff (1974) and Dallenbach-Hellweg (1975) as outlined below.

Assuming a 28 day menstrual cycle:-

Days 1-4

Menstrual, degenerating endometrium; polymorphs present.

Days 4-7

Early proliferative phase endometrium; low endometrium; freshly epithelialised basalis glands sparse, narrow and straight; loose spindly stroma; epithelial cells low columnar.

Days 7-14

Mid to late proliferative phase endometrium; increase in height of endometrium; rapidly growing glands; early tortuosity; epithelium becoming tall columnar; many mitoses in epithelial and stromal cells; early oedema evident which gradually regresses. In the later proliferative phase (days 11-14), the glands become increasingly tortuous, nuclei appear "piled up" to give the appearance of pseudo-stratification. The gland lumen becomes prominent but empty.

Days 15-28

Secretory phase (equivalent to luteal phase)

Days 15-18 (early)

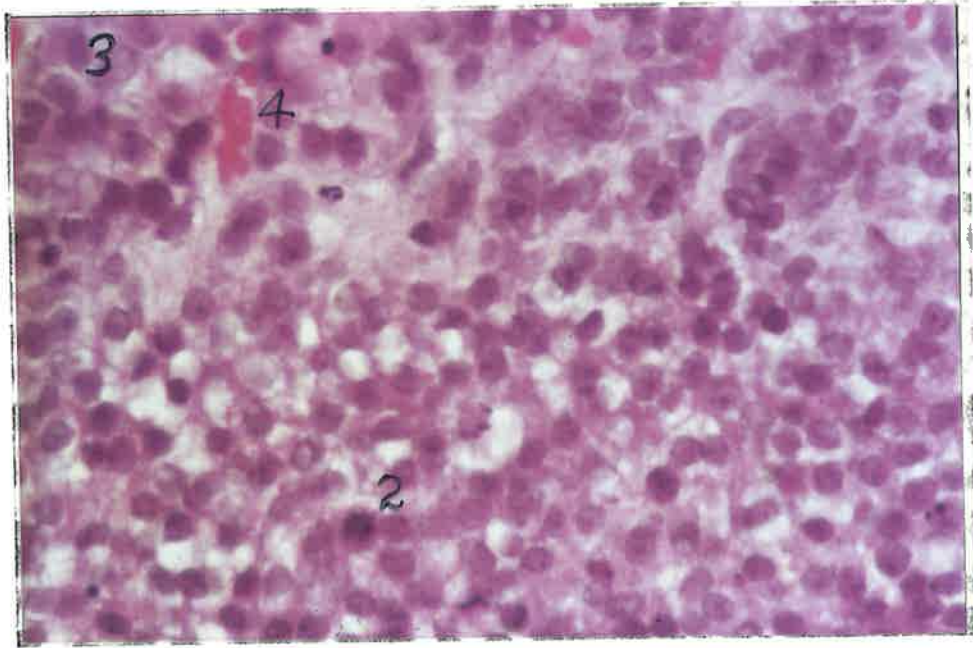
Increasing number of sub-nuclear vacuoles; increasing "cork screw" glands; atypical nuclei; very few mitoses.

Days 18-24

Nuclei return to base of cell; "secretion" bulges into gland lumen; nuclei enlarge; stromal oedema; no mitoses seen; further gland convolution; spiral arteries become prominent.

Days 25-28

Pseudo decidual change established; stromal granulocytes prominent; oedema subsides; endometrium contracts; glands collapsed and "saw toothed"; nuclear debris appears.



Photomicrograph 7. (x600)

Histological detail of follicular cells following 5 days in organ culture.

1. 90% of the cells are granulosa cells.
2. Probable mitotic figure in a granulosa cell (just after metaphase).
3. Small area of theca cells in the top left hand corner.
4. Red blood cells.

2.3. RESULTS

2.3.1. Age of patients

The average age of patients included in this section of the study was 32 years \pm 1.8 (SEM), n = 19.

Histological or Histochemical assessment following organ culture

All follicular tissue included in this study maintained good morphological characteristics as judged either histologically or histochemically. In particular there was excellent adherence of the granulosa to the theca interna following organ culture.

Incidence of infection during organ culture

Throughout all the organ culture procedures where human Graafian follicles were incubated, out of a total of two hundred and eight separate follicles, only four became infected during culture. Coincident with evidence of infection, steroid activity as judged by estimations of testosterone, oestradiol-17 β and progesterone, declined rapidly to very low levels of less than 5 ng of steroid per ml of incubation media. These results were therefore excluded from the data.

Detection of atretic or cystic follicles

Where there was a combination of very low steroid release from follicles in culture and histological evidence of marked granulosa and theca cell regression, it was decided that these follicles were non functional and therefore not representative of the follicles fulfilling a significant steroidogenic function at the time of explantation. These results were also excluded from the data.

The number of follicles which fell into this category were 27 out of the total of 208 follicles. There was no increased frequency of explanting these follicles at any particular phase of the human reproductive cycle, nor a relationship with a certain sized follicle or with age of the patient as indicated within the confines of this study.

2.3.2. Steroid profiles of early follicular phase follicles, invitro

As a general rule human follicles were most active steroidogenically during the second 24 hours in organ culture.

Steroid release, invitro, of follicles explanted during the early follicular phase of the menstrual cycle (days 1-7/28)

Testosterone was the predominant steroid released by early follicular

phase follicles when compared to oestradiol-17 β and progesterone.

Testosterone release was significantly greater than oestradiol-17 β release per mg of follicular tissue per 24 hours, ($p < 0.1$ on days 2 and 3 and < 0.01 on day 4) in organ culture, and also significantly greater than progesterone release, ($p < 0.01$ on day 1, < 0.05 on day 2, < 0.1 on day 3 and < 0.05 on day 4) in organ culture. See Table 1 and Figure 1.

2.3.3. Steroid profiles of late follicular phase follicles, invitro

Steroid release, invitro, of follicles explanted during the late follicular phase of the menstrual cycle (days 8-14/28)

Progesterone was the predominant steroid released by late follicular phase follicles when compared to oestradiol-17 β and testosterone. Progesterone release was significantly greater than testosterone release per mg of follicular tissue per 24 hours, ($p < 0.05$ on day 2, < 0.1 on day 3 and < 0.025 on day 4) in organ culture and also significantly greater than oestradiol-17 β release, ($p < 0.05$ on day 2 and 4) in organ culture. See Table 1 and Figure 2.

2.3.4. Steroid profiles of luteal phase follicles, invitro

Steroid release, invitro, from follicles explanted during the luteal phase of the menstrual cycle (days 15-28/28)

Testosterone release was significantly greater than oestradiol-17 β release per mg of follicular tissue per 24 hours, ($p < 0.1$ on day 1, < 0.05 on day 2, and < 0.025 on day 3) in organ culture. Progesterone release was also significantly greater than oestradiol-17 β release per mg of follicular tissue per 24 hours, ($p < 0.005$ on day 3 and < 0.05 on day 4) in organ culture. Progesterone and testosterone release from luteal phase follicles were comparable during the first 3 days in organ culture but progesterone release was significantly greater on day 4 ($p < 0.1$) in organ culture. See Table 1 and Figure 3.

Table 1. Release of testosterone, oestrogen and progesterone (means \pm SEM) by human ovarian follicles in organ culture explanted during the early follicular phase (days 1-7, N = 5), late follicular phase (days 8-14, n = 6) and the luteal phase (days 15-28, n = 6)

Steroid secreted into culture medium	Days after explantation	Stage of cycle and steroid output (ng of steroid/mg of follicular tissue per 24 hours)					
		Early follicular		Late follicular		Luteal	
Testosterone	1	23	\pm 3	19	\pm 2	21	\pm 5
	2	70	\pm 21	36	\pm 10	34	\pm 7
	3	41	\pm 11	22	\pm 7	22	\pm 4
	4	20	\pm 3	16	\pm 3	9	\pm 3
Oestrogen	1	14	\pm 5	30	\pm 8	19	\pm 5
	2	19	\pm 6	30	\pm 4	15	\pm 3
	3	15	\pm 5	39	\pm 7	9	\pm 2
	4	10	\pm 3	23	\pm 4	5	\pm 1
Progesterone	1	7	\pm 2	47	\pm 21	15	\pm 3
	2	9	\pm 3	119	\pm 37	18	\pm 3
	3	16	\pm 9	55	\pm 21	24	\pm 2
	4	6	\pm 3	48	\pm 10	18	\pm 5

FIGURE 1.

Spontaneous testosterone (n = 7), oestrogen (n = 6) and progesterone (n = 7) release (ng/mg of follicular tissue per 24 hours) during four days in organ culture (mean \pm SEM is shown for each day in culture), of follicles explanted during the early follicular phase (days 1-7) of the menstrual cycle.

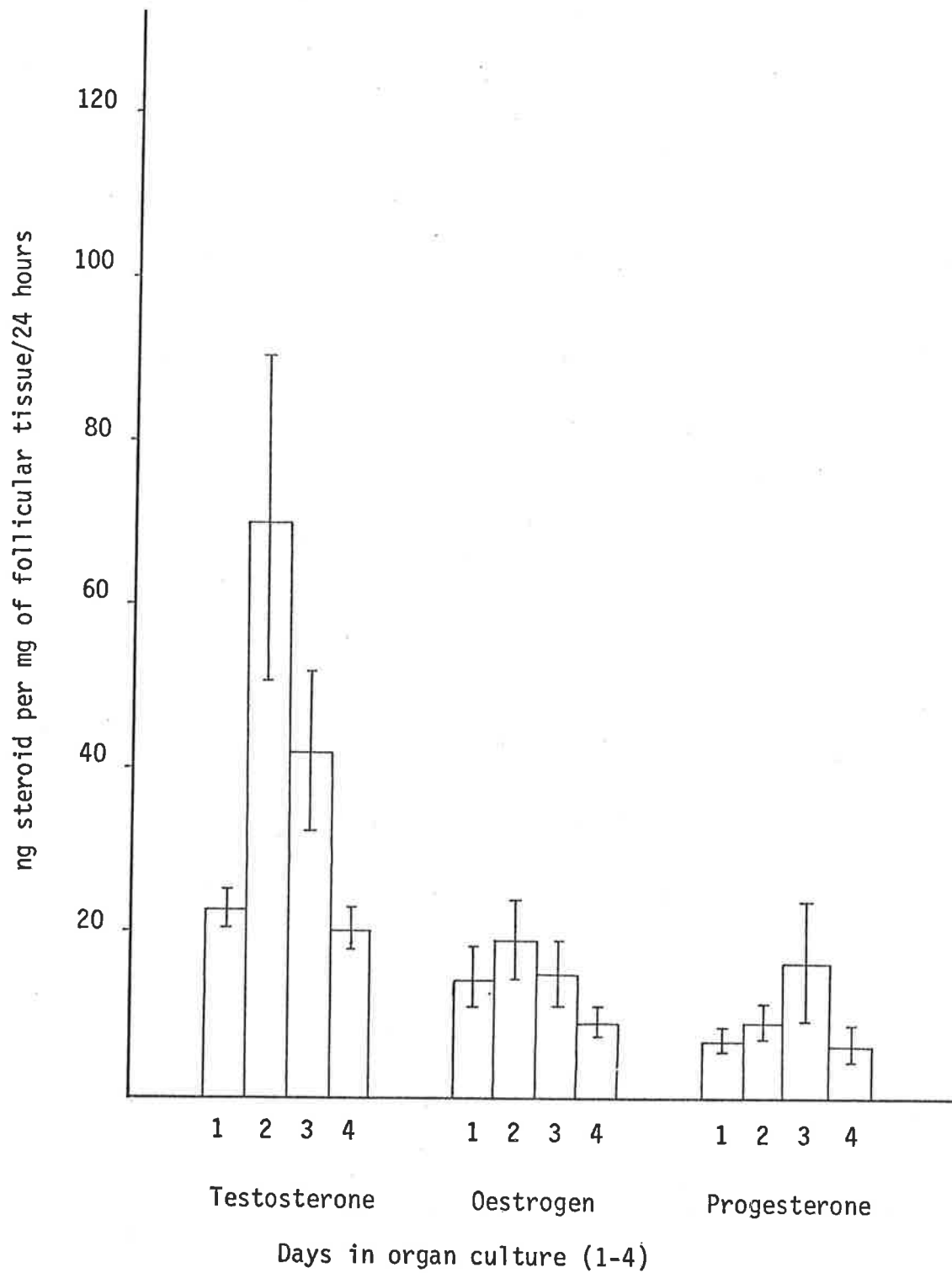
2.3.2. Steroid profiles of early follicular phase follicles invitro

FIGURE 2.

Spontaneous testosterone (n = 5), oestrogen (n = 6) and progesterone (n = 4) release (ng/mg of follicular tissue per 24 hours) during four days in organ culture (mean \pm SEM is shown for each day in culture), of follicles explanted during the late proliferative phase (days 8-14) of the menstrual cycle.

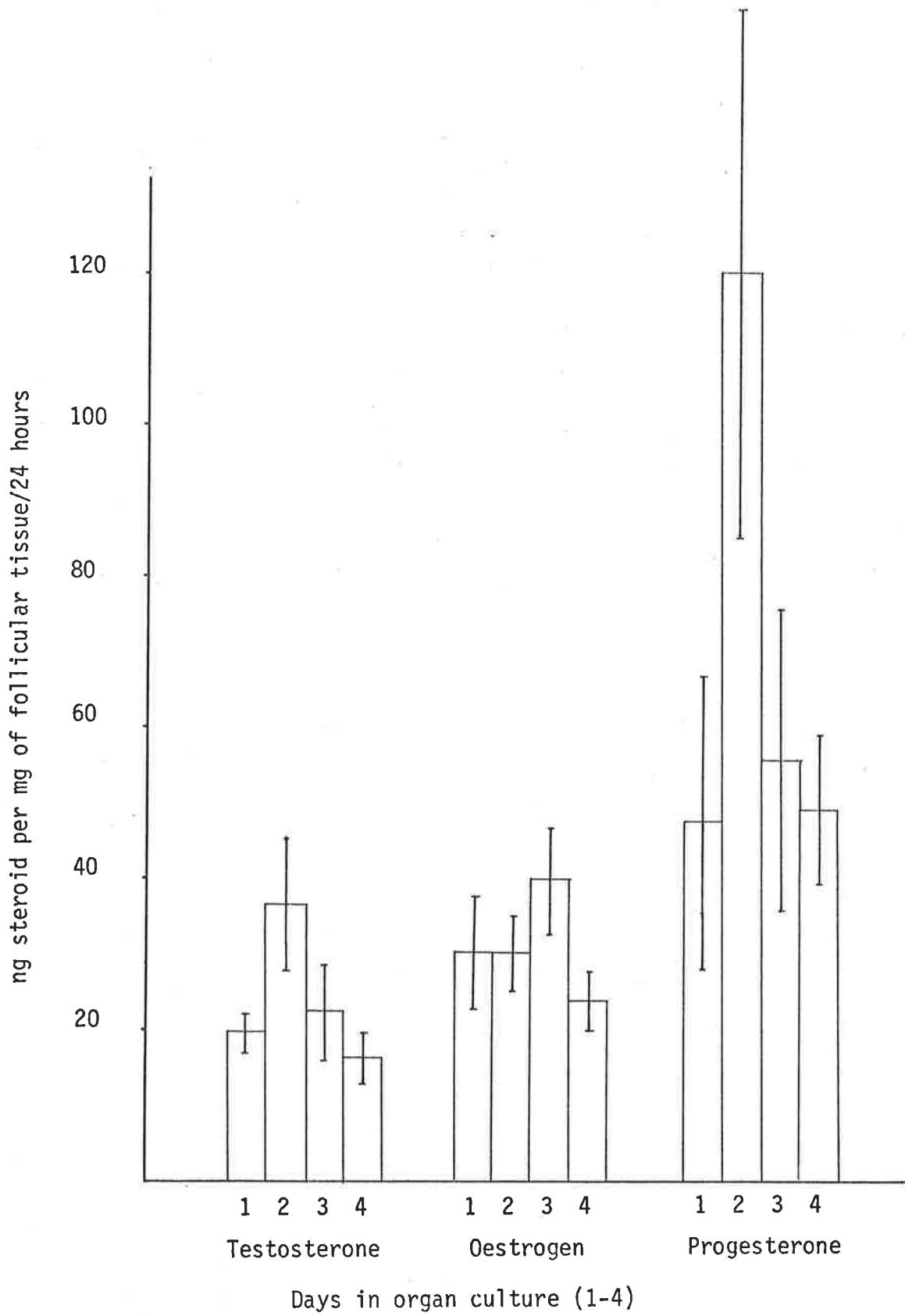
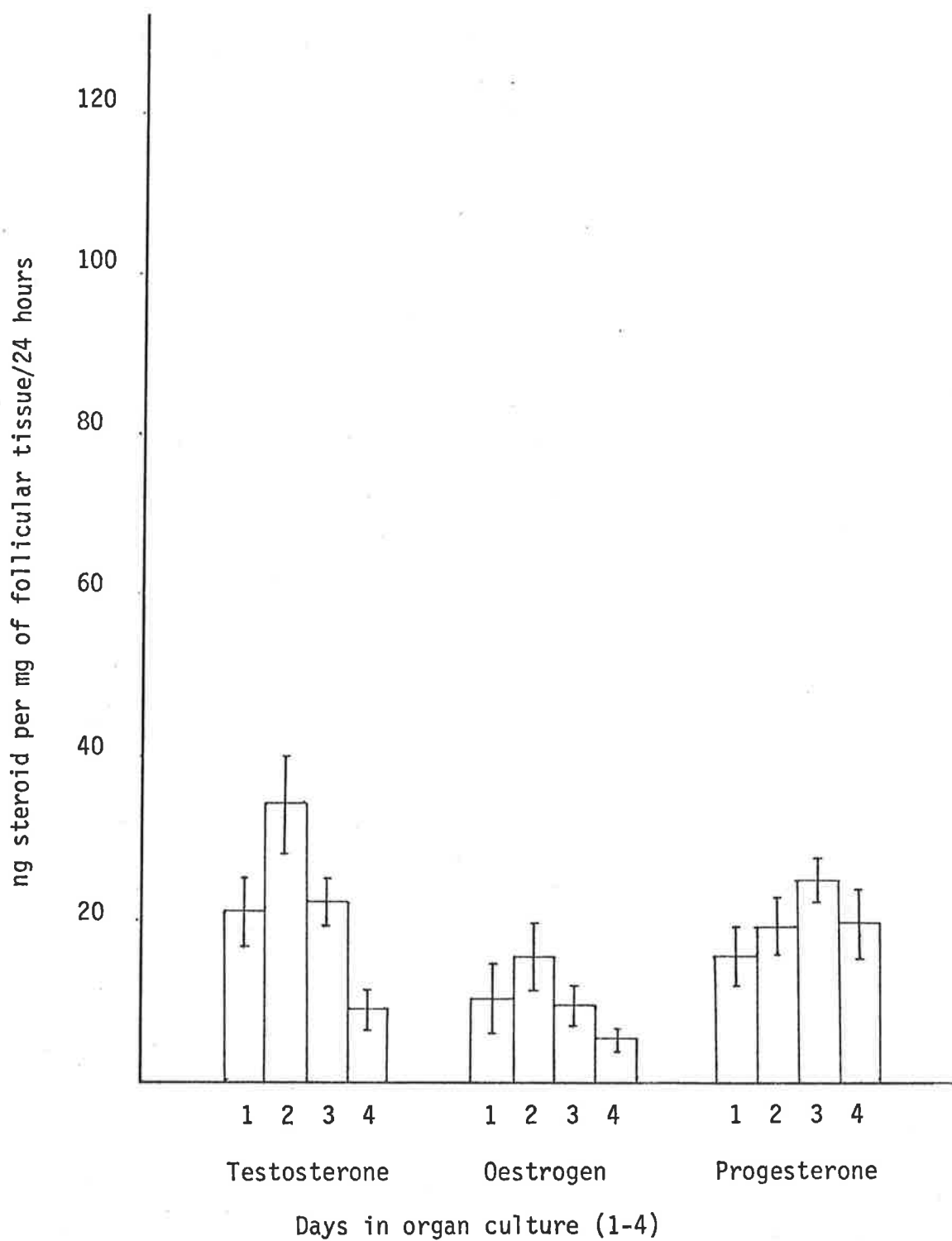
2.3.3. Steroid profiles of late follicular phase follicles invitro

FIGURE 3.

Spontaneous testosterone (n = 7), oestrogen (n = 7) and progesterone (n = 4) release (ng of steroid/mg of follicular tissue per 24 hours) during four days in organ culture (mean \pm SEM is shown for each day in culture), of follicles explanted during the luteal phase (days 15-28) of the menstrual cycle.

2.3.4. Steroid profiles of luteal phase follicles invitro

2.3.5 Testosterone profiles of follicles explanted throughout the menstrual cycle

Testosterone was the predominant steroid released in organ culture, by follicles explanted during the early follicular phase (days 1-7/28) of the menstrual cycle. Its rate of release from follicles explanted at this stage of the cycle per mg of follicular tissue per 24 hours was significantly higher than from follicles explanted during either the late follicular phase, ($p < 0.1$ on days 2 and 3 in culture), or the luteal phase, ($p < 0.1$ on days 2 and 3 and < 0.05 on day 4 in culture), of the menstrual cycle. Testosterone release by late follicular and luteal phase follicles was comparable. See Table 1 and Figure 4.

2.3.6 Oestradiol-17 β profiles of follicles explanted throughout the menstrual cycle

Oestradiol-17 β release by follicles explanted during the late follicular phase (days 8-14/28) of the menstrual cycle was double the amount released by early follicular phase follicles. ($p < 0.1$ on day 1 and < 0.025 on days 3 and 4 in culture) and three times the amount released by luteal phase follicles ($p < 0.05$ on day 1, < 0.025 on day 2 and < 0.005 on days 3 and 4 in culture) on a follicular weight for weight basis. See Table 1 and Figure 5.

2.3.7. Progesterone profiles of follicles explanted throughout the menstrual cycle

Progesterone release by follicles explanted during the late follicular phase was ten times that released by early follicular phase follicles ($p < 0.1$ on days 1 and 3, < 0.025 on day 2 and < 0.01 on day 4 in organ culture) and six times that released by luteal phase follicles ($p < 0.1$ on days 1 and 3, < 0.025 on day 2 and < 0.025 on day 4 in organ culture) on a follicular weight for weight basis. See Table 1 and Figure 6.

FIGURE 4.

Spontaneous release of testosterone (ng of steroid/mg of follicular tissue per 24 hours) during four days in organ culture (mean \pm SEM is shown for each day in culture), from follicles explanted during the early follicular (days 1-7, n = 7), late follicular (days 8-14, n = 5) and luteal phase (days 15-28, n = 7) of the menstrual cycle.

2.3.5. Testosterone profiles of follicles throughout the menstrual cycle invitro

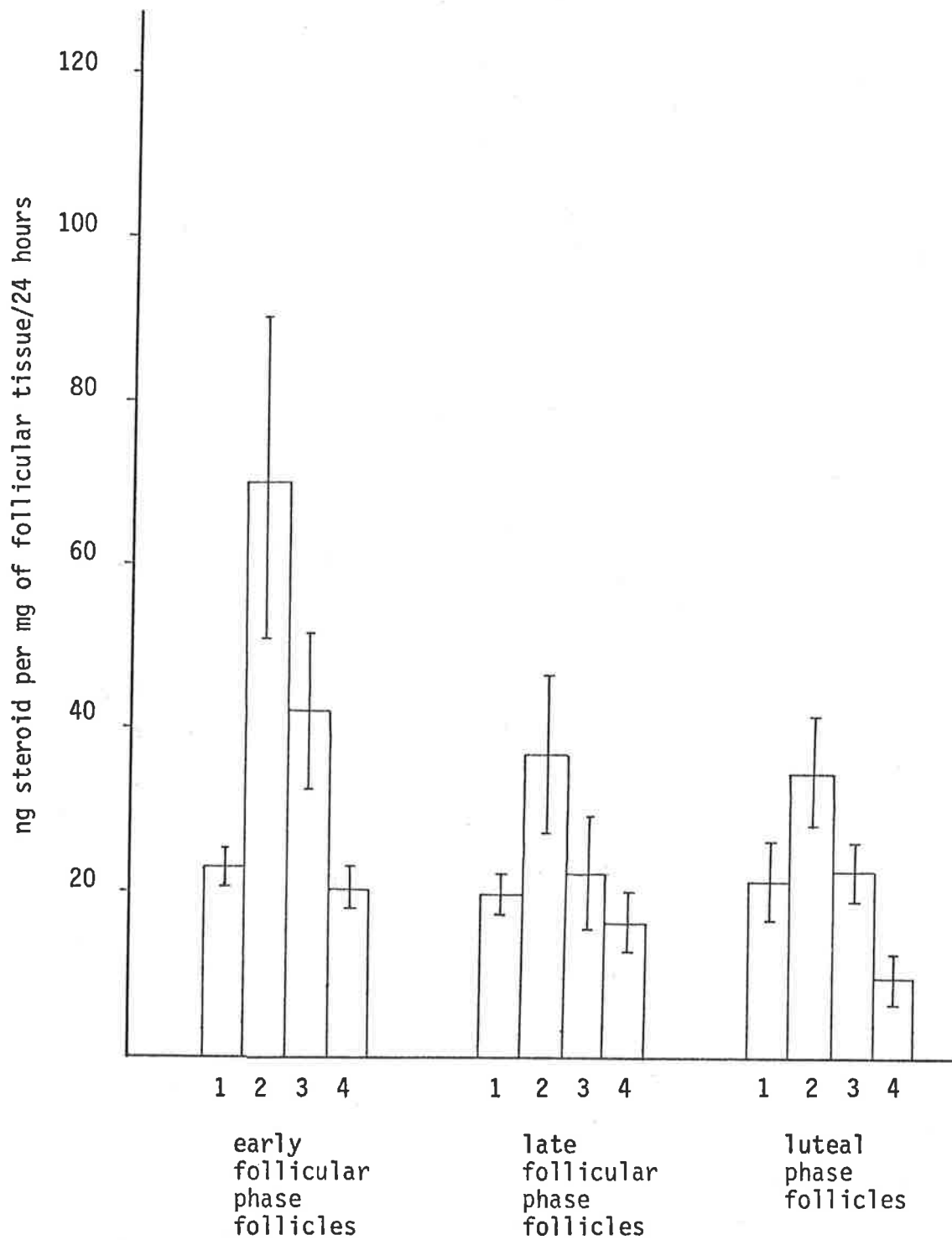


FIGURE 5.

Spontaneous release of oestrogen (ng of steroid/mg of follicular tissue per 24 hours) during four days in organ culture (mean \pm SEM is shown for each day in culture), from follicles explanted during the early follicular (days 1-7, n = 6), late follicular (days 8-14, n = 6) and luteal phase (days 15-28, n = 7) of the menstrual cycle.

2.3.6. Oestrogen profiles of follicles throughout the menstrual cycle invitro

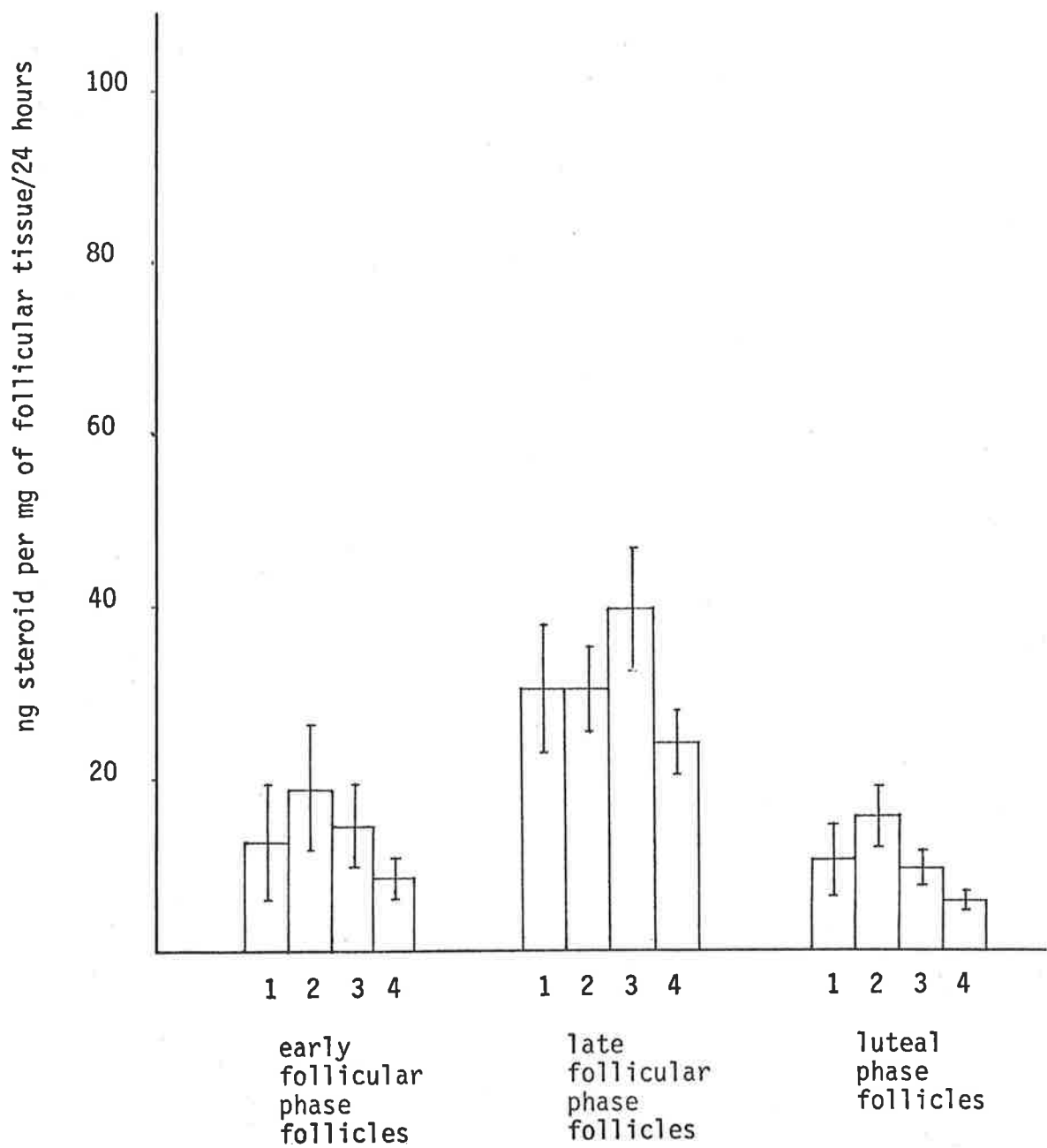
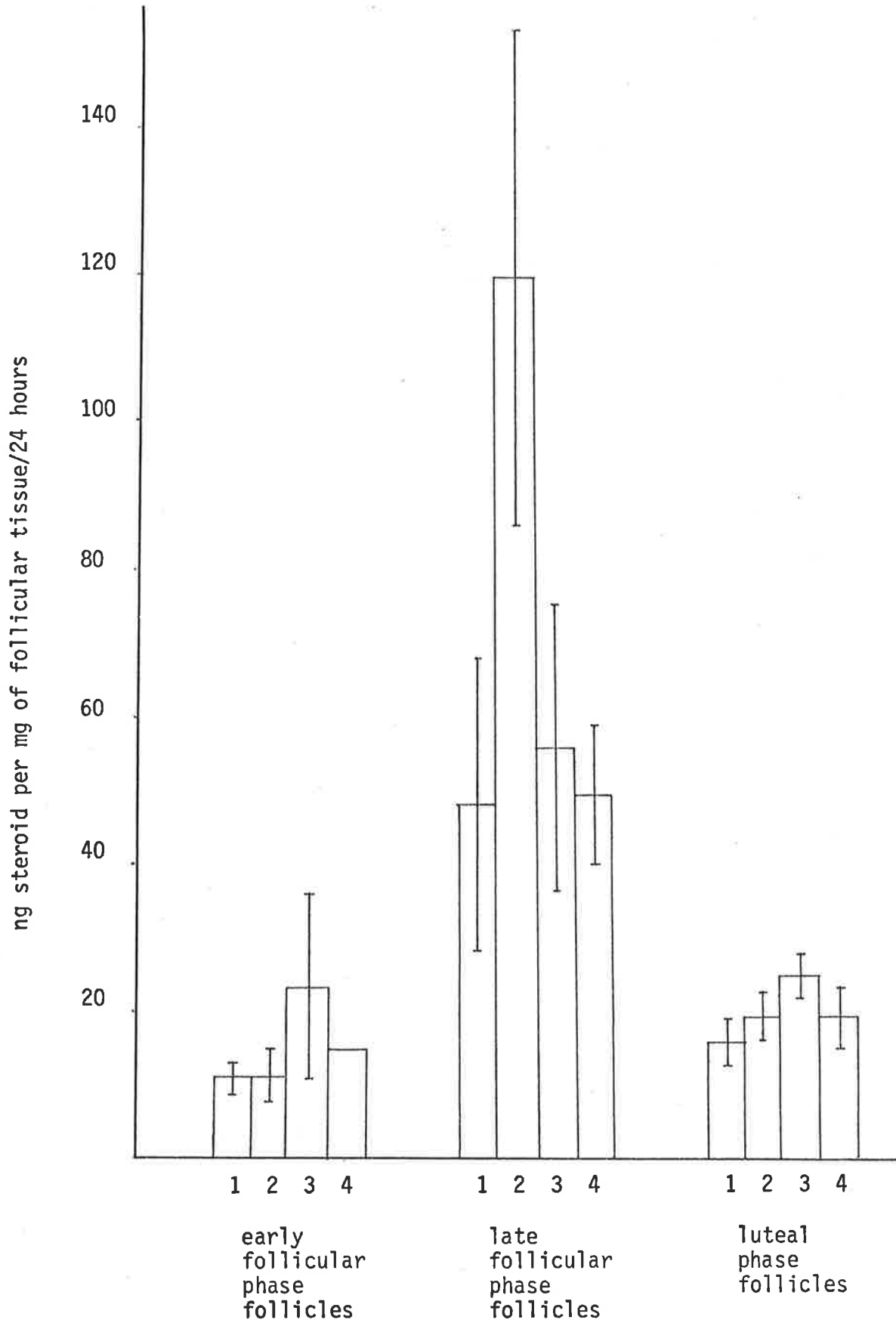


FIGURE 6.

Spontaneous release of progesterone (ng of steroid/mg of follicular tissue per 24 hours) during four days in organ culture (mean \pm SEM is shown for each day in culture), from follicles explanted during the early follicular (days 1-7, n = 6), late follicular (days 8-14, n = 4) and luteal phase (days 15-28, n = 4) of the menstrual cycle.

2.3.7. Progesterone profiles of follicles throughout the menstrual cycle invitro



2.3.8. Steroid profiles of post partum follicles

Ovarian follicles explanted two to five days after childbirth demonstrated spontaneous biosynthetic steroid activity in organ culture. See Table 2.

The follicles in this series were explanted from five women, two women were day 2, two women day 3 and one woman day 5, post partum. There was no significant difference in steroid activity or follicular size, of follicles explanted during this short post partum period.

Testosterone release by unstimulated post partum follicles was relatively high and comparable on a weight for weight basis to testosterone release by early follicular phase follicles (days 1-7/28). Oestradiol-17 β release by unstimulated post partum follicles was of the order found in late follicular phase follicles. Progesterone release by unstimulated post partum follicles was of the order found in luteal phase follicles.

Table 2. Spontaneous release of testosterone, oestradiol-17 β and progesterone (mean \pm SEM, n = 7) of human ovarian follicles in organ culture, explanted during the early post partum period (days 2-5, after parturition)

Steroid released into culture medium	Days after explantation	Steroid release in ng/mg of follicular tissue/24 h		
Testosterone	1	76	\pm	10
	2	58	\pm	9
	3	28	\pm	9
	4	5	\pm	2
Oestradiol-17 β	1	45	\pm	10
	2	33	\pm	14
	3	19	\pm	5
	4	9	\pm	3
Progesterone	1	27	\pm	7
	2	35	\pm	10
	3	27	\pm	6
	4	13	\pm	10

2.3.9. Follicular size in relation to the time of explantation throughout the reproductive cycle

In all cases the largest visible follicles were explanted at each stage of the reproductive cycle.

Table 3.

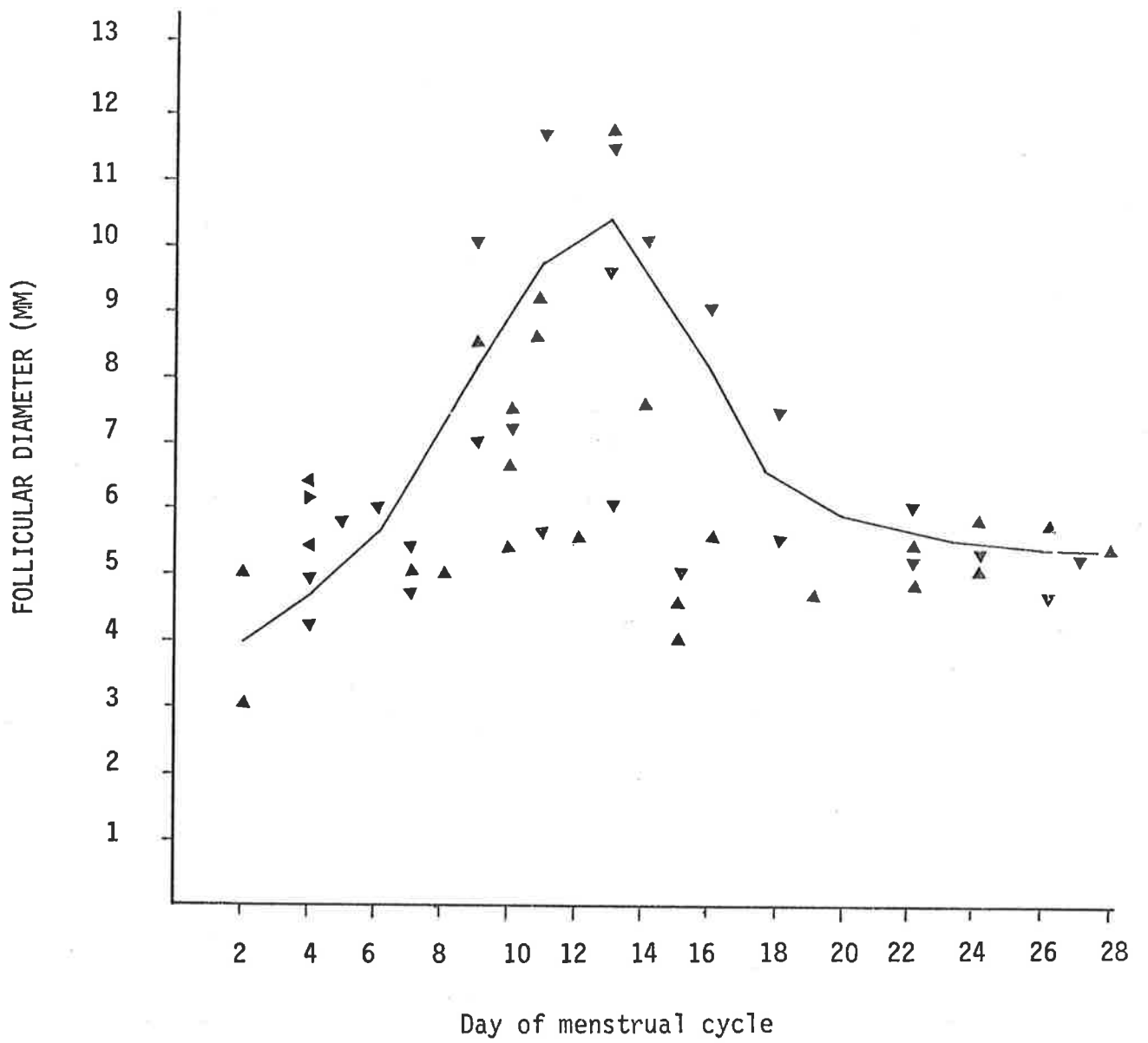
Time of explantation during the reproductive cycle	Average diameter (mm) \pm SEM
Early follicular phase follicles (days 1-7/28)	5.4 \pm 0.5 (n = 7)
Late follicular phase follicles (days 8-14/28)	8.5 \pm 0.8 (n = 6)
Luteal phase follicles (days 15-28/28)	5.5 \pm 0.5 (n = 8)
Post partum follicles (days 2-5)	4.2 \pm 0.3 (n = 8)

Graafian follicles explanted during the late follicular phase of the menstrual cycle were significantly larger than follicles explanted either during the early follicular or luteal phase ($p < 0.025$). The average diameters of early follicular and luteal phase follicles were very similar. Follicles explanted within five days of parturition were significantly smaller than both early follicular and luteal phase follicles, ($p < 0.05$).

Figure 7.

A 'Scattergram' displaying individual follicular diameters in relation to the time of explantation during the menstrual cycle is shown. Also a line illustrating the mean follicular diameter during each phase of the menstrual cycle is shown.

Figure 7.

Follicular diameter in relation to menstrual phase

2.3.10. Steroid activity in relation to the timing of explantation during the menstrual cycle

Figure 7, illustrates that the smaller early follicular phase follicles released predominantly androgens, invitro. However as the follicles matured, the follicles showed an increased ability to release both oestradiol-17 β and progesterone into the culture medium. During the late follicular phase maximal release of oestradiol-17 β and progesterone coincided with a fall in testosterone release from these larger follicles. Follicles explanted during the luteal phase demonstrated a lesser, but more consistent ability to release testosterone and progesterone but a significant decline in oestradiol-17 β release.

Maximal steroid output per unit weight of follicular tissue occurred during the late follicular phase, followed by early follicular phase and lastly luteal phase follicles. See Table 3.

Figure 8, shows that the relationship between oestradiol-17 β and testosterone, and progesterone and testosterone release, invitro, changed markedly, but in a similar reciprocal pattern throughout the menstrual cycle. In contrast a more constant relationship existed between a follicles capacity to produce oestradiol-17 β and its capacity to produce progesterone.

FIGURE 7.

Each point represents the release of testosterone (\blacktriangle), oestrogen (\bullet) and progesterone (\square) by individual human ovarian follicles during 4 days in organ culture, in relation to the time of explantation during the menstrual cycle.

2.3.10. The relationship between steroid production of ovarian follicles in culture to the day of the menstrual cycle 79

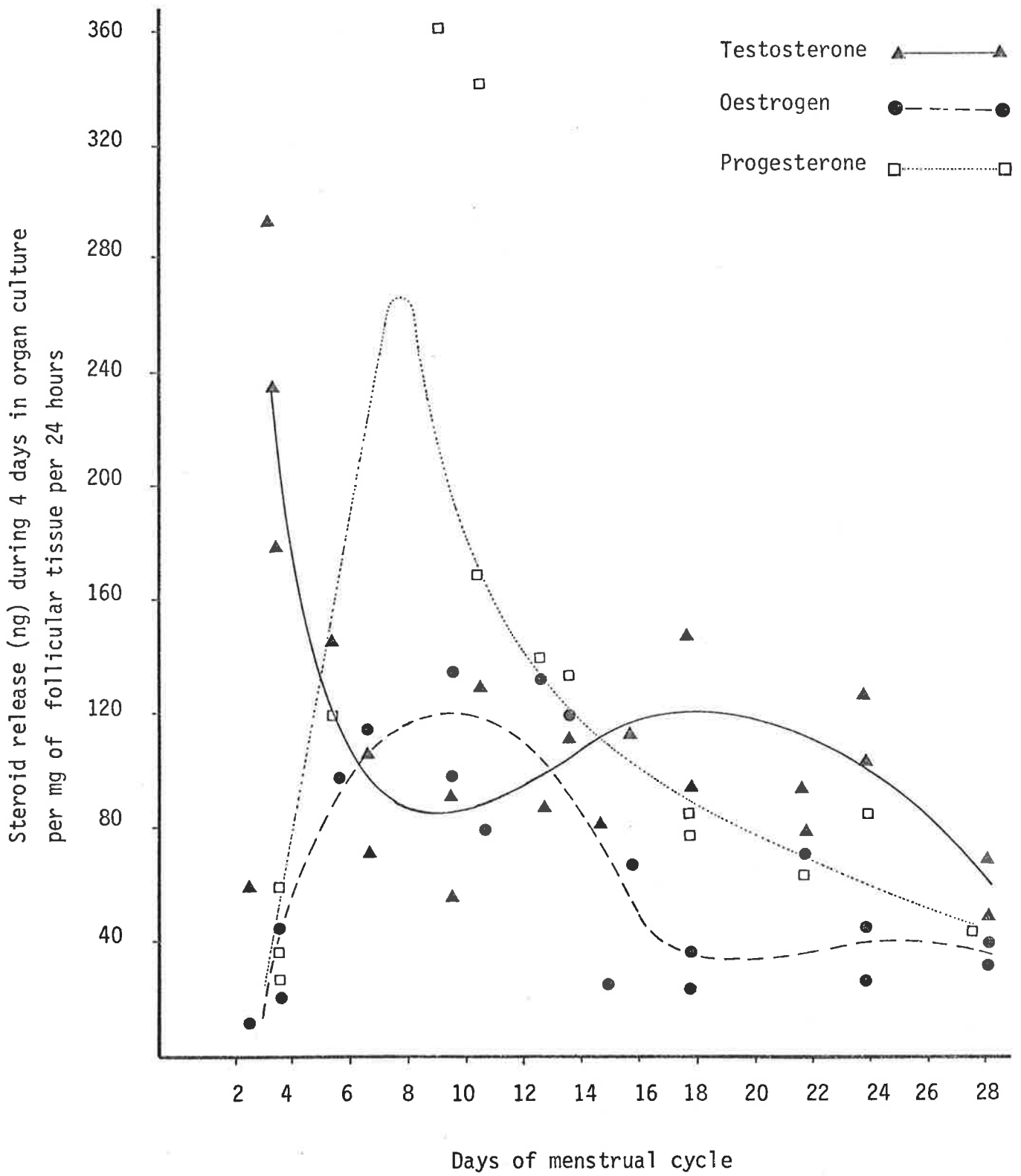


FIGURE 8.

Graphs 1-4.

Interrelationship of the steroids, testosterone, oestrogen and progesterone released by ovarian follicles invitro, in relation to the time of explantation within the menstrual cycle. Each point represents a ratio derived for each follicle by using the following formula:-

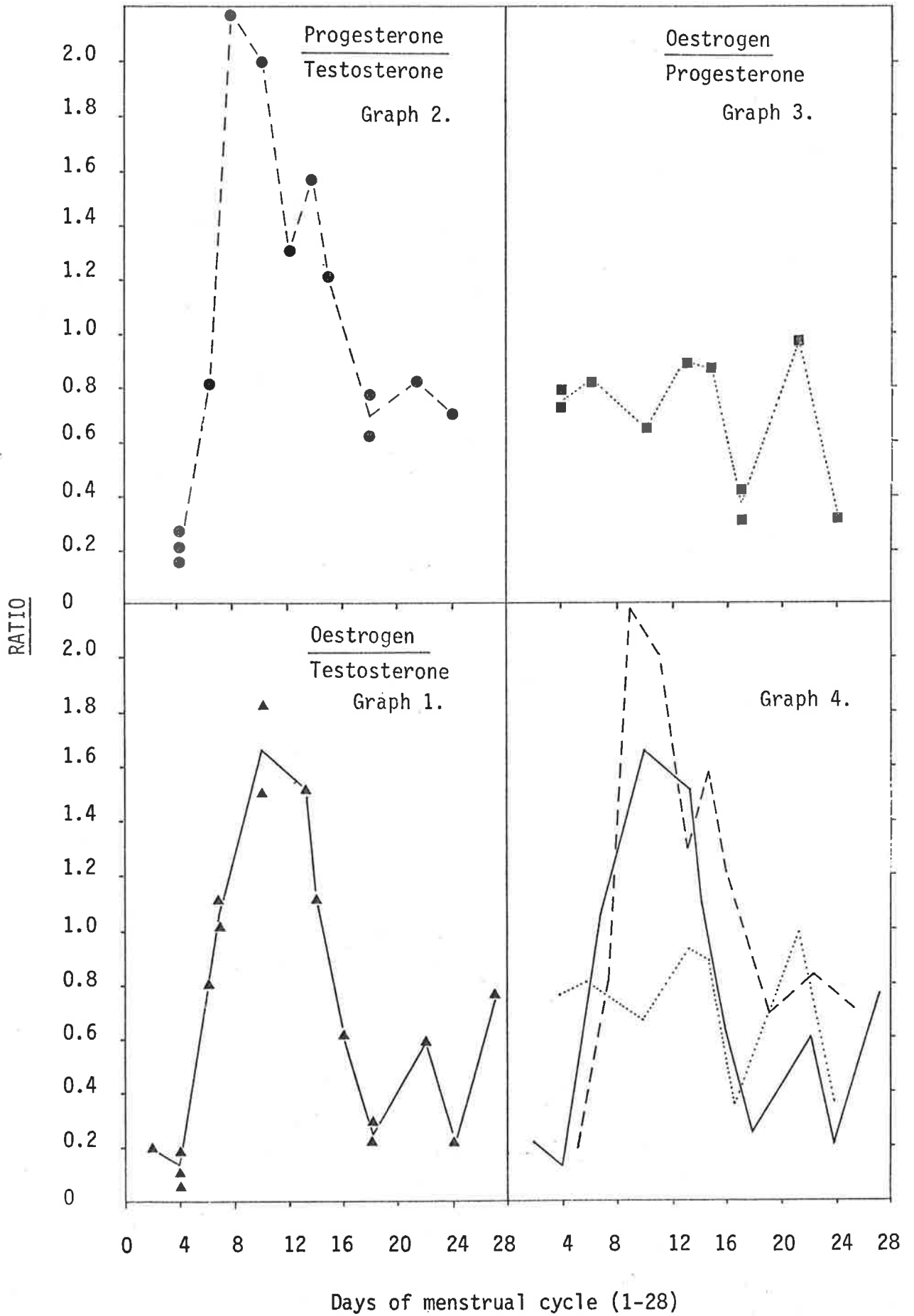
average steroid (1) release (ng)
over 4 days in culture, per mg of
follicular tissue per 24 hours.

average steroid (2) release
(ng) over 4 days in culture,
per mg of follicular tissue
per 24 hours.

Graph 1. (E/T) and Graph 2.(P/T) shows that a reciprocal relationship exists between oestrogen and testosterone and also progesterone and testosterone production throughout the menstrual cycle. i.e. E/T and P/T are <1 in the early follicular phase, >1 in the late follicular phase and again <1 during the luteal phase.

Graph 3. (E/P) on the other hand shows a constant interdependent relationship between a follicle's capacity to produce progesterone follows its capacity to produce oestrogen. i.e. E/P is <1 throughout the menstrual cycle.

Graph 4. Composite graph.



2.3.11. Steroid activity in relation to follicular diameter

It has been demonstrated that steroid activity per unit of follicular weight does vary in relation to follicular diameter independent of the time of explantation within the menstrual cycle. Figure 9, illustrates that there is a steady increase in testosterone, oestradiol-17 β and progesterone release as the follicle grows to a diameter of 6-8 mm. Beyond this diameter there is a gradual decrease in both testosterone and to a lesser extent progesterone, but a continual rise in oestradiol-17 β release. One qualification that must be noted is that follicles of more than 8 mm in diameter only occur during the preovular phase of the menstrual cycle and so at this stage, follicular size invariably relates to the time of explantation within the menstrual cycle.

The summation of testosterone, oestradiol-17 β and progesterone activity appears to be maximal in relation to follicular weight in the 6-8 mm follicle, (see figure 9).

2.3.12. Steroid activity in relation to follicular size and the time of explantation during the menstrual cycle

As illustrated in Figure 10, there are marked differences in steroid activity of similar sized follicles at different stages of the menstrual cycle. The most obvious example being in relation to progesterone release which is maximal from follicles of 6-8 mm in diameter, explanted during the late follicular phase of the menstrual cycle.

FIGURE 9.

Average release of testosterone (\blacktriangle), oestrogen (\bullet) and progesterone (\blacksquare) by individual follicles over 4 days in organ culture, expressed as ng of steroid per mg of follicular tissue per 24 hours. These values are plotted against follicular diameter independent of the time of explantation during the menstrual cycle. The graphs for each steroid are derived from the average steroid release from follicles of a particular diameter.

Total steroid (testosterone + oestrogen + progesterone) release (ng) per mg of follicular tissue in relation to follicular diameter is also shown (\circ).

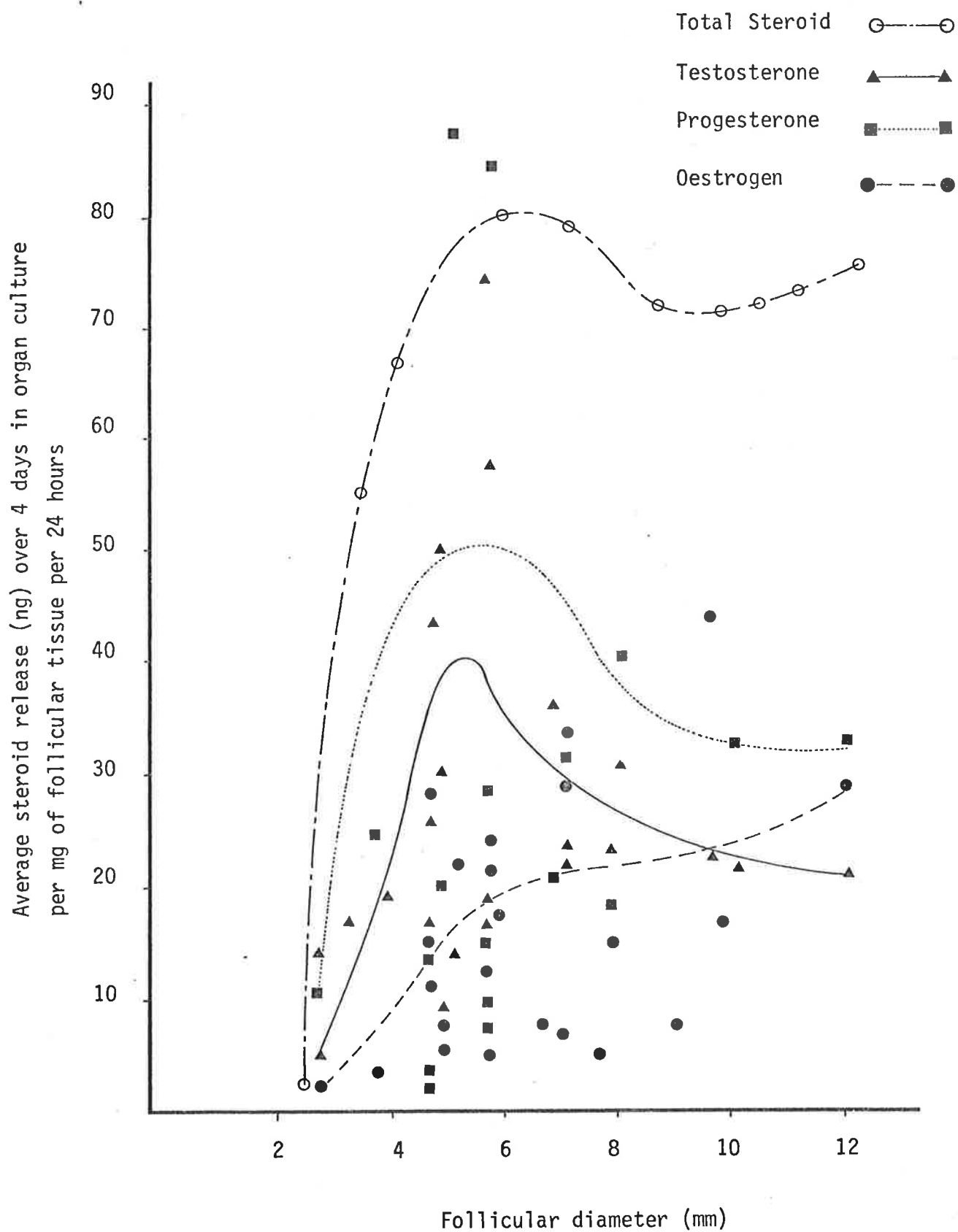
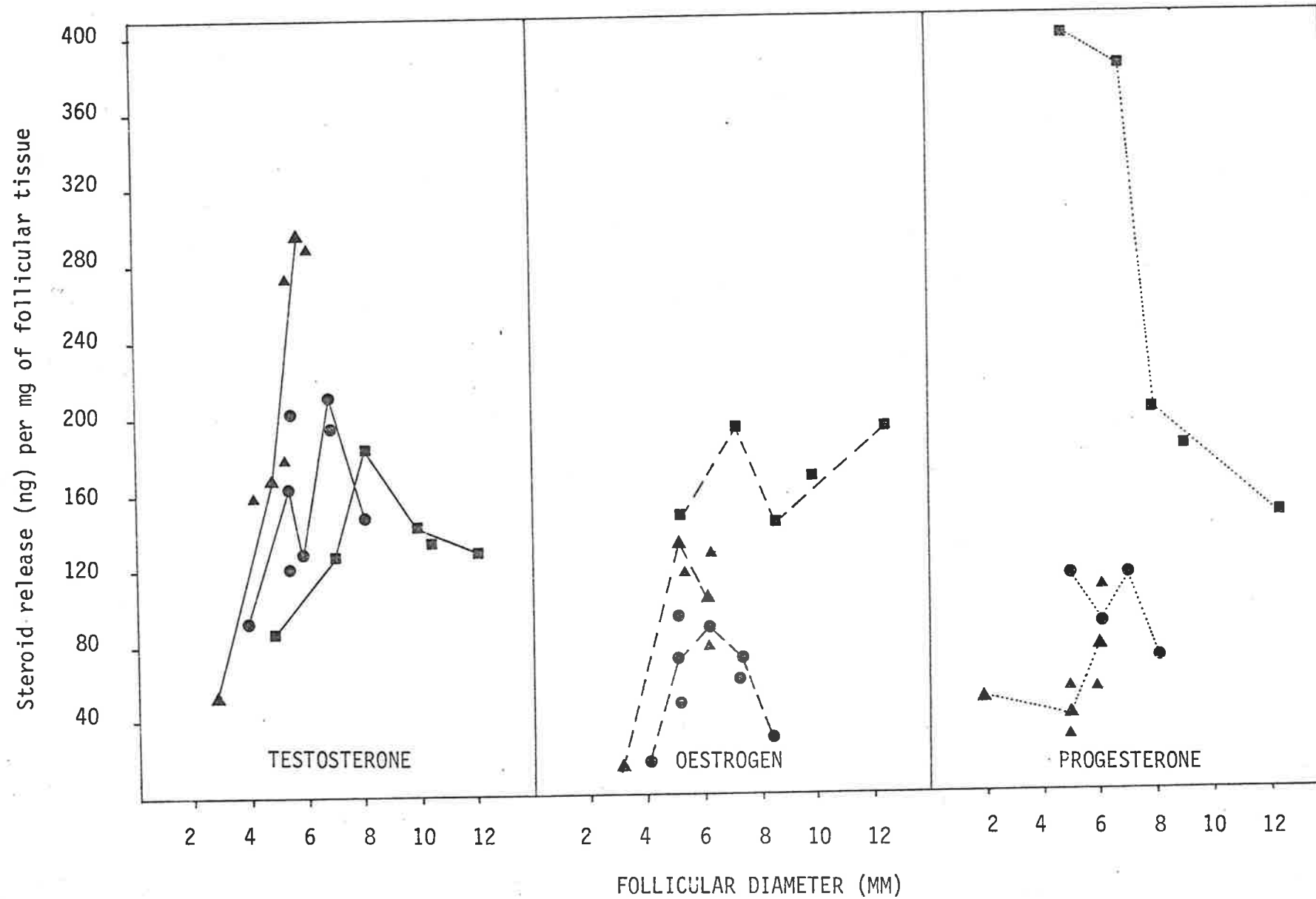


FIGURE 10.

The relationship between testosterone, oestrogen and progesterone activity during 4 days in organ culture in relation to follicular diameter and explantation during the early follicular phase (▲), late follicular phase (■) and the luteal phase (●).

The results indicate that steroid activity of human ovarian follicles, invitro, is related to at least two factors, one being follicular size and the other the time in the menstrual cycle when explantation took place.

Steroid activity in relation to follicular diameter and menstrual phase



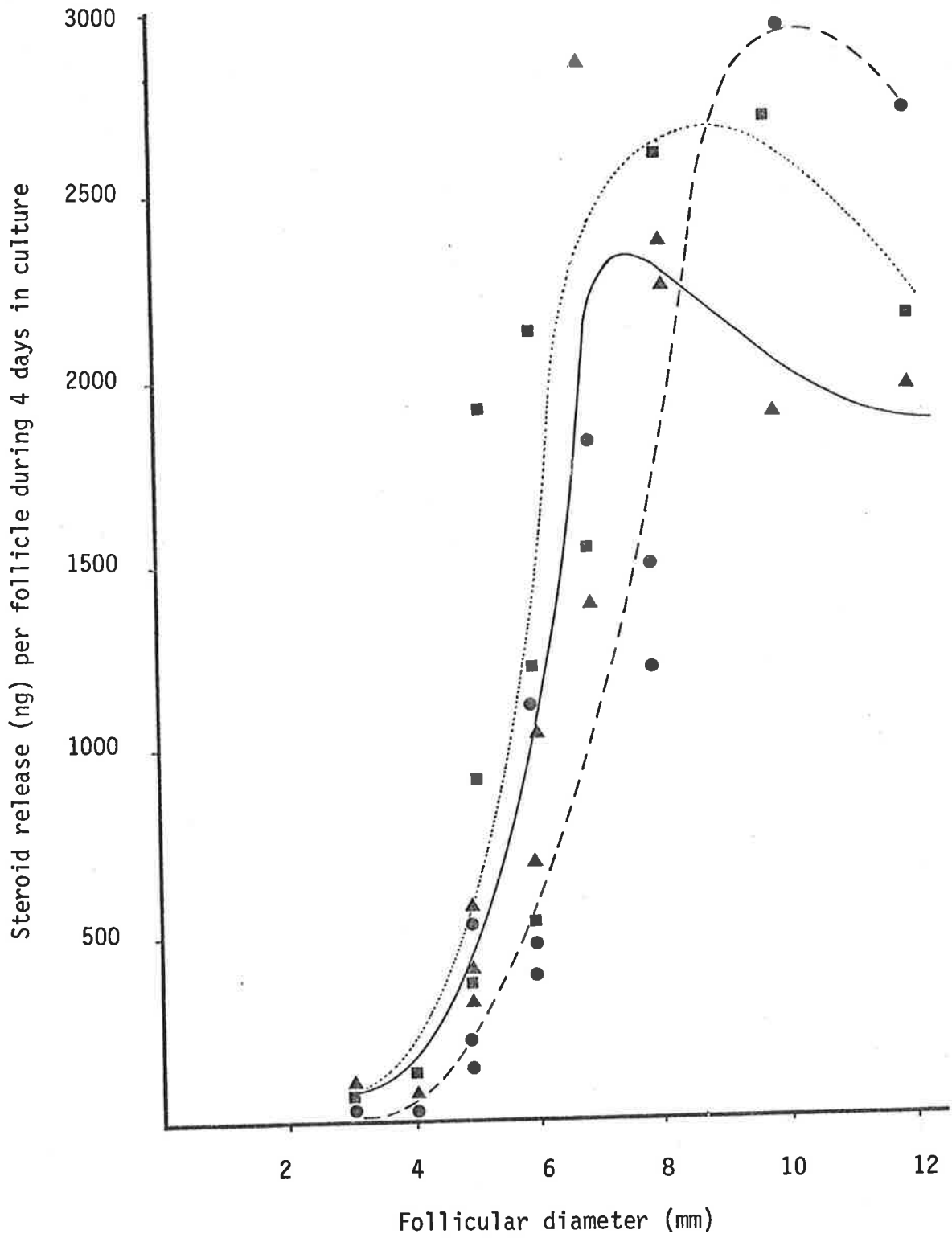
2.3.13. Steroid activity of late follicular phase follicles in relation to their diameter

In this experiment steroid activity of whole follicles, invitro, was studied from follicles which were only explanted during the late follicular phase of the menstrual cycle (days 8-14/28). Also steroid release is measured (see Figure 11) from each follicle in relation to its diameter and not as ng of steroid per mg of follicular tissue. It can be seen that there is a rapid increase in testosterone, oestradiol-17 β and progesterone in an almost parallel fashion from follicles ranging from 3 to 8 mm in diameter and then a fall off in firstly testosterone, then progesterone and lastly oestradiol-17 β release from follicles of 8 to 12 mm in diameter. However steroid release by these large follicles is still high.

On one occasion the explantation of the only large (11.5 mm diameter) preovular follicle (day 12 of a regular 28 day cycle) resulted in a rapid fall in blood oestradiol-17 β levels within one hour of explantation and also slight uterine withdrawal bleeding which began 24 hours later and lasted for 2 days. This follicle was very active in culture and histologically normal after culture.

FIGURE 11.

Release of testosterone (▲), oestrogen (●) and progesterone (■) by individual follicles which were explanted during the late follicular phase of the menstrual cycle (days 8-14) during 4 days in organ culture, in relation to their diameter.



2.4. DISCUSSION

The results of this study confirms the feasibility of maintaining isolated human ovarian follicles in organ culture to facilitate meaningful and detailed endocrine investigations. Histological integrity was maintained throughout the culture period and the incidence of infection of follicles in culture was very low.

Endocrine function as assessed by steroid release was sustained in culture but varied between individual follicles and was related to size and the stage in the menstrual cycle when explantation took place. Similar findings were noted for cultured sheep follicles, (Seamark, Moor and McIntosh, 1974) and cultured rabbit ovarian follicles. (Younglai, 1977). The pattern of steroid release by isolated human follicles in culture tended to follow the steroid profiles found in follicular fluid at various stages of the menstrual cycle, (Kemeter, Salzer, Breitenacker and Friedrich, 1975).

It has been demonstrated that human follicles explanted during the early follicular, late follicular, luteal phase and the early post partum period of the reproductive cycle are all able to release the three basic sex steroids, testosterone, oestradiol-17 β and progesterone, invitro.

By only explanting the largest visible follicles at the time of ovarian biopsy the pattern of follicular growth throughout the reproductive cycle could be evaluated. It was found the late follicular phase follicles were significantly larger than either early follicular or luteal phase follicles. Early follicular and luteal phase follicles were comparable in size. Furthermore, follicles explanted within five days of parturition were significantly smaller than both early follicular and luteal phase follicles. These observations lend support to the hypothesis proposed by Pedersen, (1970), that follicular growth, once started is a continuum, and continues until they either become atretic or ovulate. The observation that only follicles greater than 8 mm in diameter occurred during the late follicular phase supports the concept suggested by Govan, (1968, 1970) that a sharp distinction between the continuum of follicular growth and the cyclic event with consequent rapid growth during the periovular period must be made. The acquisition of functional gonadotrophin receptor sites by the theca and granulosa cells has been correlated with follicular maturation (Lunenfeld, Kraiem and Eshkol, 1975; Nimrod, Erickson and Ryan, 1976; Stouffer, Tyrey and Schomberg, 1976). A logical sequence of events appears to occur. As the destined

group of follicles for a particular menstrual cycle grow, their progressive release of oestradiol-17 β into the blood enhances pituitary storage of gonadotrophins. During the preovular period when oestradiol-17 β levels are high pituitary LH becomes releasable (Knobil, 1974, Yen, 1975) and at the same time the selected late follicular phase follicle, or follicles, having undergone particular maturation changes, have functional receptor sites and the hormonal environment for optimal response to gonadotrophins with consequent rapid follicular growth.

Ovarian follicles procured early in the follicular phase of the menstrual cycle produced predominantly testosterone in organ culture; presumably from the theca cells. Supportive evidence for this premise has been found by the observation that the enzyme 3 β -HSD activity is almost exclusively confined to the theca of these follicles, (see chapter 4), and that isolates of theca and granulosa cells, *invitro*, release testosterone, mainly from the theca cell isolates, (see chapter 5).

Follicles harvested during the late follicular phase were more active steroidogenically on a weight for weight basis than follicles explanted at any other stage of the human reproductive cycle. Also, the pattern of steroid release changed when compared to the smaller early follicular phase follicle, in that they produced more oestradiol-17 β and progesterone, but less testosterone in culture. As noted by McKay, Pinkerton, Hertig and Danziger, (1961), histologically human follicular structure is similar in follicles ranging from 0.3 to 10 mm in diameter. That observation has been confirmed in this study. However from a steroidogenic and histochemical point of view, changes occur in relation to both follicular size and the hormonal environment influencing follicular function at the time of explantation. It has been clearly demonstrated that similar sized follicles explanted at different stages of the menstrual cycle have varying capacities to produce steroids. Also follicles explanted during the late follicular phase of the menstrual cycle demonstrate a variable steroid pattern in relation to their diameter. It therefore appears that steroid activity of follicles *invitro*, must be considered in relation to two independent variables, follicular size and the time of follicular explantation during the menstrual cycle, (see figure 10).

It has been shown that there is a rapid and progressive increase in the release of testosterone, oestradiol-17 β and progesterone from follicles ranging from 3 to 8 mm in diameter. A gradual decline in

firstly testosterone, then progesterone and oestradiol-17 β release occurs from follicles of 8-12 mm in diameter, (see figure 11). These findings suggest that in the course of accelerated follicular growth prior to ovulation both the aromatizing enzyme and the 3 β -HSD enzyme in the granulosa layer, (see chapter 4), are activated. This activation results in a sustained rise in oestradiol-17 β production by the large preovular follicle possibly at the expense of the substrate precursor, testosterone. Interestingly the capacity of a follicle to produce progesterone in culture was correlated to its ability to produce oestrogen, indicating that synthesis of both steroids may be dependent on maturational changes in the granulosa layer. This concept was supported by finding that the ratio of oestradiol-17 β /progesterone production was fairly constant for follicles explanted throughout the menstrual cycle. By contrast a reciprocal relationship existed between oestradiol-17 β /testosterone and progesterone/testosterone production, (see figure 8). The sustained release of progesterone by these late follicular phase follicles correlates with the histochemical localization of 3 β -HSD activity in the 'luteinized' granulosa cells of these follicles in culture (see chapter 4). Observations on human preovular follicular fluid, (Kometer et al., 1975), the preferential use of the Δ^4 -ketone pathway by human granulosa cells, (Ryan and Petro, 1966), the histochemical detection of 3 β -HSD in the granulosa cells of preovular follicles, (Friedrich et al., 1974) and the steroid activity of human granulosa cell isolates, invitro, (Channing, 1969), tend to support the observations made.

Follicles explanted during the luteal phase were less active steroidogenically on a weight for weight basis than early follicular phase follicles, but very similar in size. McNatty, Hunter, McNeilly and Sawers (1975), were unable to find large active follicles in the ovary of women during the luteal phase. In contrast to late follicular phase follicles the relationship between oestradiol-17 β and testosterone was reversed in that testosterone release was significantly greater than oestradiol-17 β . This observation is consistent with the hypothesis that aromatase activity may be declining. Baird and Frazer (1975), also noted that human luteal phase follicles secreted little oestrogen, and that follicles greater than 5 mm in diameter underwent degeneration. Further evidence for this premise was found from incubating luteal phase follicles with gonadotrophins (see chapter 3). Progesterone release by luteal phase follicles was greater than that released by early follicular phase follicles. This finding coupled with the observations

that progesterone release by luteal phase follicles was significantly increased during incubation with both FSH and LH (see chapter 3) and that 3β -HSD enzyme activity was maintained in the granulosa cells (see chapter 4) supports the hypothesis that the granulosa cells of luteal phase follicles retain significant steroidogenic and enzyme activity.

Follicles explanted from days 2 to 5 following parturition were significantly smaller than any other groups studied, invitro. These follicles spontaneously released all three groups of sex steroids, invitro, in amounts more comparable to follicles obtained during the follicular than the luteal phase of the menstrual cycle. Blood prolactin levels were measured in these women at the time of follicular explantation and were found to be higher (see chapter 6) than values noted by Tyson, Khojanai, Huth and Adreassen, (1975) and Rolland et al (1975), for post partum women in the first week following parturition. It has been noted (see chapter 6) that surgical stress increases blood prolactin levels very significantly and could account for the high values observed. Unfortunately follicular fluid prolactin levels were not measured in this study. This would have been of value, especially in light of the observations by McNatty, Sawers and McNeilly, (1974), that high levels of follicular prolactin may be inhibitory to progesterone production. Prolactin levels were measured in culture media from post partum follicles and found to be within the range found for follicles explanted during the menstrual cycle (see chapter 6). Despite the post partum follicle's ability to spontaneously release steroids in culture, their response to incubation with gonadotrophins was minimal, (see chapter 3).

2.5. SUMMARY

The feasibility of maintaining isolated human follicles in organ culture to facilitate useful endocrine investigations has been achieved. Observations indicate that follicular growth is a continuum, but that rapid follicular growth only occurs during the late follicular phase of the reproductive cycle. Endocrine function as assessed by steroid release was sustained in culture but varied between individual follicles and was related to both their size and the stage in the menstrual cycle when explantation took place.

Follicles obtained during the late follicular phase of the menstrual cycle (days 8-14/28) of mean diameter 8.5 mm were the most active steroidogenically and continued to release oestrogen, testosterone and progesterone throughout the 4 day period of culture. Both the total

and relative amounts of steroid released by individual late follicular phase follicles varied with follicular size. Testosterone production was maximal in follicles of about 8 mm diameter, whereas larger follicles released less androgen but more oestrogen and progesterone in culture.

Follicles obtained earlier in the cycle (days 1-7/28) were smaller, with a mean diameter of 5.4 mm, and produced less oestrogen and progesterone, but more androgen per mg of follicular tissue than the late follicular phase follicles. Follicles obtained during the luteal phase (days 15-28/28) of the menstrual cycle produced similar amounts of androgen but less oestrogen and progesterone than the late follicular phase follicles. Follicles obtained from ovaries within the first week following parturition were the smallest group cultured with a mean diameter of 4.2 mm, and produced all three steroids in amounts more comparable to follicles obtained during the follicular than luteal phase of the menstrual cycle.

CHAPTER THREE

3. STEROID PROFILES OF HUMAN OVARIAN FOLLICLES IN ORGAN CULTURE DURING INCUBATION WITH GONADOTROPHINS. CORRELATIONS WITH FOLLICLES REMOVED IN THE EARLY FOLLICULAR, LATE FOLLICULAR, LUTEAL PHASE AND THE IMMEDIATE POST PARTUM PERIOD

3.1. INTRODUCTION

The role of the gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) on follicular maturation has been extensively investigated, *in vivo* and *in vitro*. The recent literature review (pages 8-16) covers important work done by investigators in this field. The correct interplay between the developing follicle and the hypothalamic pituitary axis via released oestradiol-17 β , and in turn the controlled cyclical release of gonadotrophins from the anterior pituitary to influence follicular maturation via specific receptor sites is essential.

An attempt has been made to examine the different effects FSH and HCG has on follicles explanted at different stages of the reproductive cycle.

3.2. MATERIALS & METHODS

3.2.1. The Gonadotrophins used

The follicle stimulating hormone (FSH) used was obtained from the Commonwealth Serum Laboratories (Australia). The preparation was obtained from that fraction of the human pituitary gland which contained most of the FSH activity. It was noted that this was not a pure preparation of FSH and as stated by the manufacturers contained a variable amount of LH. A dose of 20 iu of FSH per ml of culture medium was used. The potency of this preparation was measured by the Steelman-Pohley bioassay against the 2nd International Reference Preparation of Human Menopausal Gonadotrophin as standard.

The luteotrophic hormonal preparation used was Pregnyl - Chorionic gonadotrophin, B.P. (LH), obtained from Organon Laboratories (England). This preparation was used in a dosage of 20 iu/ml of culture medium.

3.2.2. Incubation procedure

The techniques of follicular biopsy, microdissection and incubation were identical to those outlined in the methodology section of Chapter 2. The gonadotrophins were added to the culture media from day one of

incubation. The culture media was aspirated every 24 hours and the fresh media contained the same dose of either FSH or LH throughout the incubation period (usually 4 days). On all occasions the follicles were assessed histochemically, for the localization of 3β -HSD activity (see Chapter 4).

3.3. RESULTS

3.3.1. The differential steroid activity of early follicular, compared to late follicular phase follicles during incubation with gonadotrophins

The effect of including gonadotrophins in the culture media on steroid release by follicles is shown in Table 1, and Figures 1, 2 & 3.

The incubation of early follicular phase follicles with HCG resulted in a marginally significant increased release of testosterone ($p < 0.1$ on day 1 in culture), and a very significant increased release of progesterone ($p < 0.025$ on days 1 and 2, < 0.05 on day 3 and < 0.1 on day 4 in culture), and no significant change in oestradiol- 17β release, when compared to control follicles.

The incubation of late follicular phase follicles with FSH resulted in a significant increased release of testosterone ($p < 0.1$ on day 2 in culture), a significant increased release of oestradiol- 17β ($p < 0.1$ on day 2 in culture) and a significant increased release of progesterone ($p < 0.1$ on day 3 in culture), when compared to control follicles.

The incubation of late follicular phase follicles with HCG resulted in a significant increased release of testosterone ($p < 0.05$ on day 1 and < 0.1 on day 2 in culture), and a significant reduction in the release of oestradiol- 17β ($p < 0.05$ on days 1, 2, 3 and 4 in culture) and no significant change in progesterone release, when compared to control follicles.

Both early and late follicular phase follicles were able to increase testosterone release by a small yet significant amount when incubated with HCG. Insufficient data was available to evaluate the effect of incubating early follicular phase follicles with FSH. The incubation of early follicular phase follicles with HCG did not alter the already low release of oestradiol- 17β by these follicles, but it did have a significant inhibitory effect on oestradiol- 17β release by late follicular phase follicles. By contrast the incubation of late follicular phase follicles with FSH was associated with a significant increased

release of oestradiol-17 β . Despite contamination of the FSH preparation with HCG, it enhanced oestradiol-17 β release by late follicular phase follicles, whereas incubation of these follicles with HCG was associated with an inhibition of oestradiol-17 β release. (See Table 1). Incubation of early follicular phase follicles with HCG, was associated with a significant increased release of progesterone, however incubation of late follicular phase follicles with HCG did not augment progesterone release. Incubation of late follicular phase follicles with FSH, however did augment progesterone release. The ability of HCG to cause functional, luteinization of the granulosa cells of early follicular phase follicles, invitro, was supported by the demonstration of 3 β -HSD activity in these follicles following culture when compared to unstimulated control follicles, (see Chapter 4). In retrospect I would like to point out that the control follicles were significantly larger than those treated with HCG ($p < 0.1$) in the group explanted in the late follicular phase of the menstrual cycle, (see Table 1). This fact may partly explain why these smaller late follicular phase follicles failed to augment their progesterone output during incubation with HCG, because they may have been the follicles less likely to proceed to ovulation if left invivo.

3.3.2. The differential steroid activity of luteal phase follicles during incubation with either FSH or HCG

The incubation of luteal phase follicles with FSH was not associated with a significant change in the release of either testosterone or oestradiol-17 β , however it was associated with a significant increased release of progesterone ($p < 0.05$ on days 1 and 2 and < 0.1 on day 3 in culture), when compared to control follicles. (See Table 2).

The incubation of luteal phase follicles with HCG was associated with a small but significant increased release of testosterone ($p < 0.1$ on day 1 in culture) and progesterone ($p < 0.1$ on days 1, 2 and 3 in culture), but had no significant effect on oestradiol-17 β release when compared to control follicles, see Figures 4, 5 & 6.

In both follicular and luteal phase follicles, incubation with HCG was associated with a greater testosterone output compared to incubation with FSH. Neither FSH or HCG had a significant effect on oestradiol-17 β activity in luteal phase follicles, whereas they had significant but opposite effects on the release of oestradiol-17 β by late follicular phase follicles. Both FSH and HCG were associated with an increased

release of progesterone by luteal phase follicles, however incubation with FSH was associated with a greater release of progesterone. Similarly FSH was associated with a significant increase in the release of progesterone by late follicular phase follicles, yet HCG was associated with a marked release of progesterone by early follicular phase follicles. It would be interesting to note the effect of incubating early follicular phase follicles with FSH.

The diameters of luteal phase follicles in the control, FSH treated and HCG treated groups were comparable, (see Table 2). This observation was in marked contrast to follicles explanted in the late follicular phase and gives further support to the thesis that rapid follicular growth occurs at this stage of the menstrual cycle.

3.3.3. Steroid activity of post partum follicles during incubation with FSH

The incubation of ovarian follicles explanted during the first week following parturition with FSH did not significantly alter their release of either testosterone or oestrogen when compared to unstimulated control follicles. However, incubation of these follicles with FSH was associated with a transient, yet significant increased release of progesterone ($p < 0.1$ on day 1 in culture). The diameters of post partum follicles in both the control group and the group treated with FSH were comparable (see Table 3).

Table 1. Release of testosterone, oestradiol-17 β and progesterone (mean \pm SEM) during incubation of early follicular phase follicles with HCG (n = 4) as compared to matched controls (n = 5), and late follicular phase follicles with FSH (n = 5) and HCG (n = 6) as compared to matched controls (n = 6)

Steroid released into culture medium	Days after explanation	Steroid output in ng/mg of follicular tissue per 24 hours														
		Early Follicular					Late Follicular									
		Control		HCG 20 iu/ml medium			Control		FSH 20 iu/ml medium		HCG 20 iu/ml medium					
Testosterone	1	23	\pm	3	48	\pm	16 *	19	\pm	2	25	\pm	6	63	\pm	23 **
	2	70	\pm	21	97	\pm	39	36	\pm	10	91	\pm	25 *	85	\pm	27 *
	3	41	\pm	11	48	\pm	18	22	\pm	7	38	\pm	17	35	\pm	7
	4	20	\pm	3	24	\pm	8	16	\pm	3	28	\pm	16	(20)		-
Oestradiol-17 β	1	14	\pm	5	13	\pm	3	30	\pm	8	22	\pm	5	7	\pm	4 **
	2	19	\pm	6	24	\pm	6	30	\pm	4	156	\pm	67 *	12	\pm	3 **
	3	15	\pm	5	10	\pm	4	39	\pm	7	46	\pm	17	6	\pm	1 **
	4	10	\pm	3	7	\pm	3	23	\pm	4	31	\pm	11	4	\pm	2 **
Progesterone	1	7	\pm	2	52	\pm	18 ***	47	\pm	21	19	\pm	5	69	\pm	31
	2	9	\pm	3	104	\pm	25 ***	119	\pm	37	147	\pm	41	92	\pm	26
	3	16	\pm	9	50	\pm	9 **	55	\pm	21	146	\pm	53 *	49	\pm	3
	4	6	\pm	4	22	\pm	8 *	48	\pm	10	68	\pm	12	37	\pm	8
Average diameter of follicles (mm)		5.4	\pm	0.5	4.6	\pm	0.5	8.5	\pm	0.8	7.1	\pm	1.1	6.1	\pm	0.4 *

The significant differences in steroid activity of follicles incubated with gonadotrophins compared to untreated control follicles is shown by asterisks, * p<0.1, ** p<0.05 and *** p<0.025.

Table 2. Release of testosterone, oestradiol-17 β and progesterone (mean \pm SEM) during incubation of luteal phase follicles with FSH (n = 3) and HCG (n = 5), as compared to matched controls (n = 6)

Steroid released into culture medium	Days after explanation	Steroid output in ng/mg of follicular tissue per 24 hours		
		Control	FSH 20 iu/ml medium	HCG 20 iu/ml medium
Testosterone	1	21 \pm 5	14 \pm 8	56 \pm 14 *
	2	34 \pm 7	27 \pm 23	64 \pm 23
	3	22 \pm 4	35 \pm 15	22 \pm 8
	4	9 \pm 3		5 \pm 2
Oestradiol-17 β	1	10 \pm 5	34 \pm 17	24 \pm 10
	2	15 \pm 3	34 \pm 17	19 \pm 13
	3	9 \pm 2	11 \pm 5	22 \pm 7
	4	5 \pm 1		8 \pm 4
Progesterone	1	15 \pm 3	148 \pm 54 **	78 \pm 25 *
	2	18 \pm 3	156 \pm 54 **	53 \pm 11 *
	3	24 \pm 2	69 \pm 18 *	91 \pm 28 *
	4	18 \pm 5		
Average diameter of follicles (mm)		5.5 \pm 0.5	5.3 \pm 0.2	5.0 \pm 0.1

Significant differences from controls are denoted as for Table 1.

Table 3. Release of testosterone, oestrogen and progesterone (means \pm SEM) from human ovarian follicles in organ culture explanted during the post partum period (days 2-4, after parturition). Steroid production from unstimulated follicles (n = 7) and FSH stimulated follicles (n = 3) are shown.

Steroid secreted into culture medium	Days after explantation	Steroid output in ng of steroid/mg of follicular tissue per 24 hours	
		Control	FSH (20 iu/ml of medium)
Testosterone	1	76 \pm 10	72 \pm 9
	2	58 \pm 9	72 \pm 6
	3	28 \pm 9	54 \pm 14
	4	5 \pm 2	27 \pm 8
Oestrogen	1	45 \pm 10	24 \pm 9
	2	33 \pm 14	18 \pm 9
	3	19 \pm 5	13 \pm 5
	4	9 \pm 3	7 \pm 3
Progesterone	1	27 \pm 7	103 \pm 41 *
	2	35 \pm 10	62 \pm 32
	3	27 \pm 6	45 \pm 18
	4	13 \pm 10	29 \pm 8
Average diameter of follicles		4.2 \pm 0.3	4.1 \pm 0.5

Significant differences from controls are denoted as for Table 1.

FIGURE 1.

Comparisons of testosterone release (ng/mg of follicular tissue per 24 hours) during 4 days in organ culture (mean \pm SEM is shown for each day in organ culture), of follicles explanted during the early follicular phase (days 1-7), during incubation:-

(1) without the addition of gonadotrophin (control),
n = 5

(2) during incubation with HCG, n = 4

and of follicles explanted during the late follicular phase (days 8-14), during incubation:-

(1) without the addition of gonadotrophin (control),
n = 6

(2) during incubation with FSH, n = 5

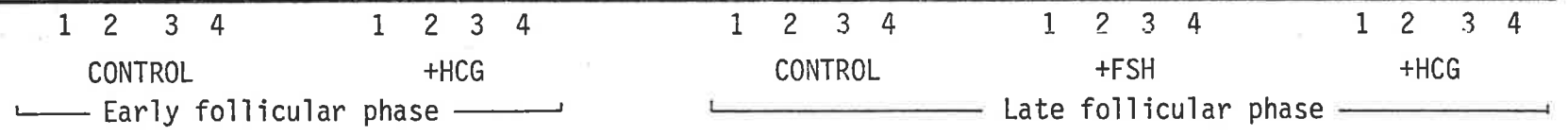
(3) and during incubation with HCG, n = 6

The significant differences in steroid activity of follicles incubated with gonadotrophins compared to untreated control follicles is shown by asterisks, * $p < 0.1$, ** $p < 0.05$ and *** $p < 0.025$.

ng of testosterone per mg of follicular tissue/24 hours



Jays in organ culture (1-4)



*

*

**

*

FIGURE 2.

Comparisons of oestradiol-17 β release (ng/mg of follicular tissue per 24 hours) during 4 days in organ culture (mean \pm SEM is shown for each day in organ culture), of follicles explanted during the early follicular phase (days 1-7), during incubation:-

(1) without the addition of gonadotrophin (control),
n = 5

(2) during incubation with HCG, n = 4

and of follicles explanted during the late follicular phase (days 8-14), during incubation:-

(1) without the addition of gonadotrophin (control),
n = 6

(2) during incubation with FSH, n = 5

(3) and during incubation with HCG, n = 6

* Notation of significant differences are as shown in the legend of Figure 1.

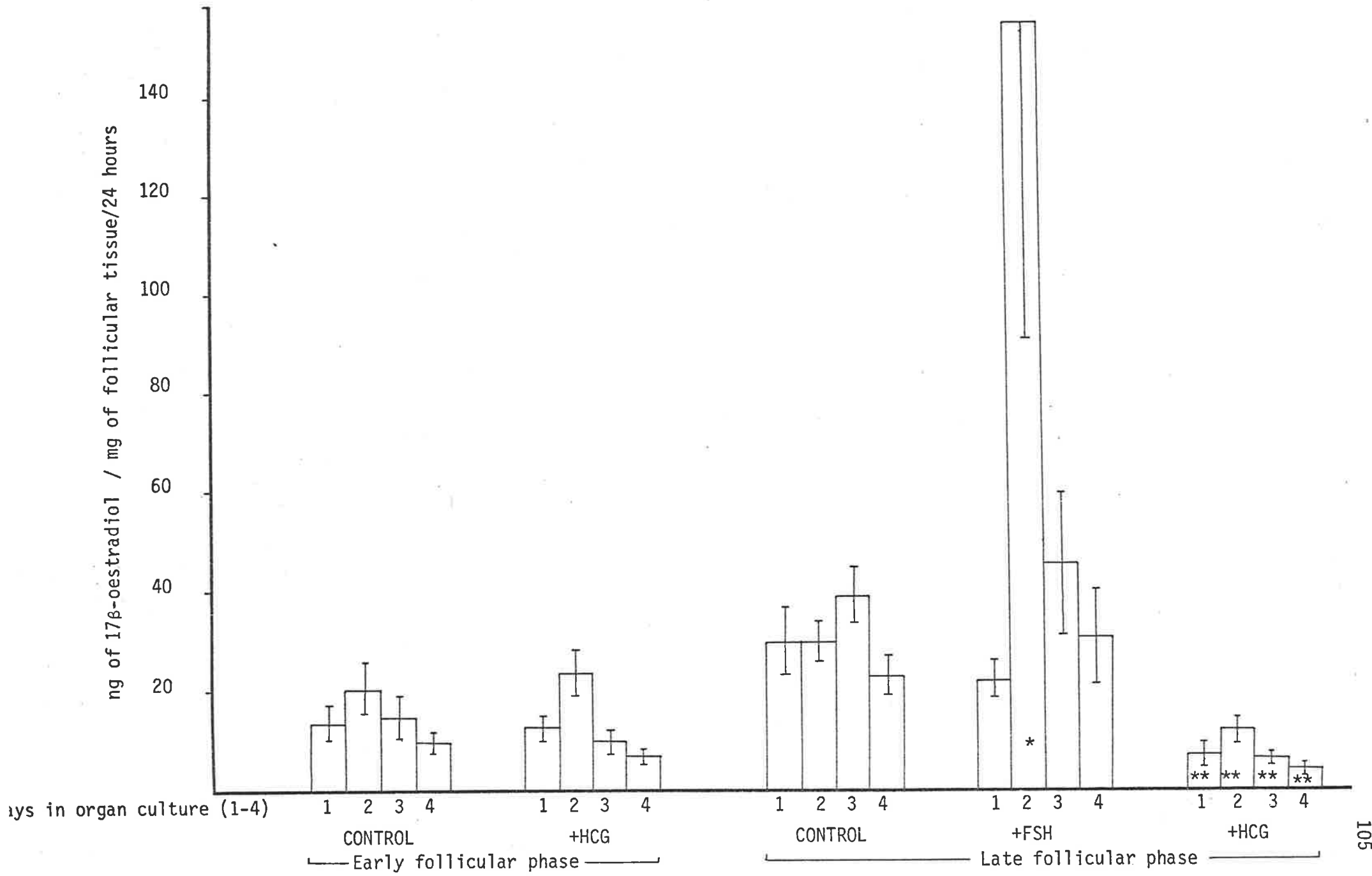


FIGURE 3.

Comparisons of progesterone release (ng/mg of follicular tissue per 24 hours) during 4 days in organ culture (mean \pm SEM is shown for each day in organ culture), of follicles explanted during the early follicular phase (days 1-7), during incubation:-

(1) without the addition of gonadotrophin (control),
n = 5

(2) during incubation with HCG, n = 4

and of follicles explanted during the late follicular phase (days 8-14), during incubation:-

(1) without the addition of gonadotrophin (control),
n = 6

(2) during incubation with FSH, n = 5

(3) and during incubation with HCG, n = 6

* Notation of significant differences are as shown in the legend of Figure 1.

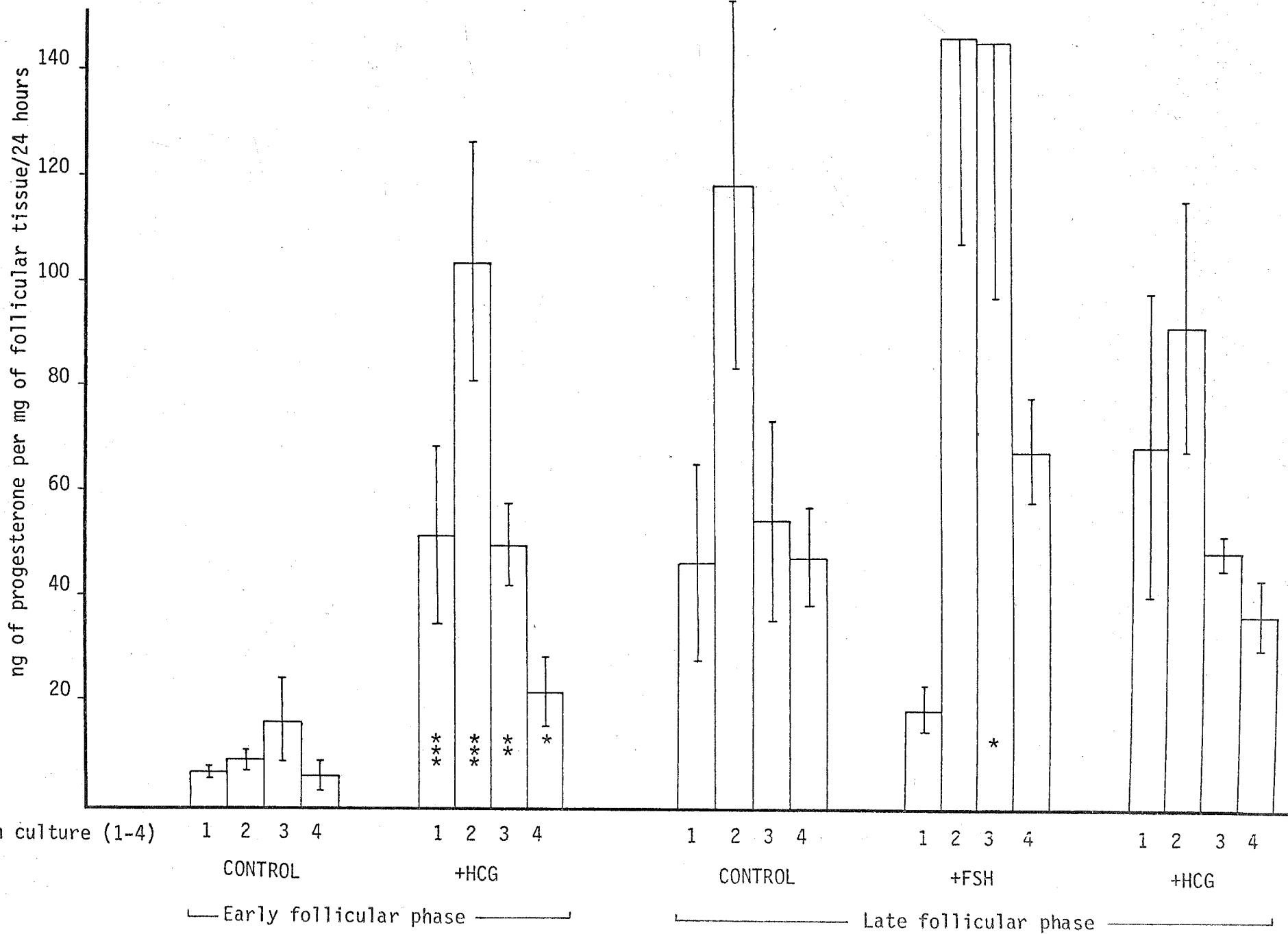


FIGURE 4.

Comparisons of testosterone release (ng/mg of follicular tissue per 24 hours) during 4 days in organ culture (mean \pm SEM is shown for each day in organ culture), during incubation:-

- (1) without the addition of a gonadotrophin (n = 7)
- (2) during incubation with FSH (n = 3)
- (3) during incubation with HCG (n = 5)

These follicles were explanted during the luteal phase (days 15-28) of the menstrual cycle.

* Notation of significant differences are as shown in the legend of Figure 1.

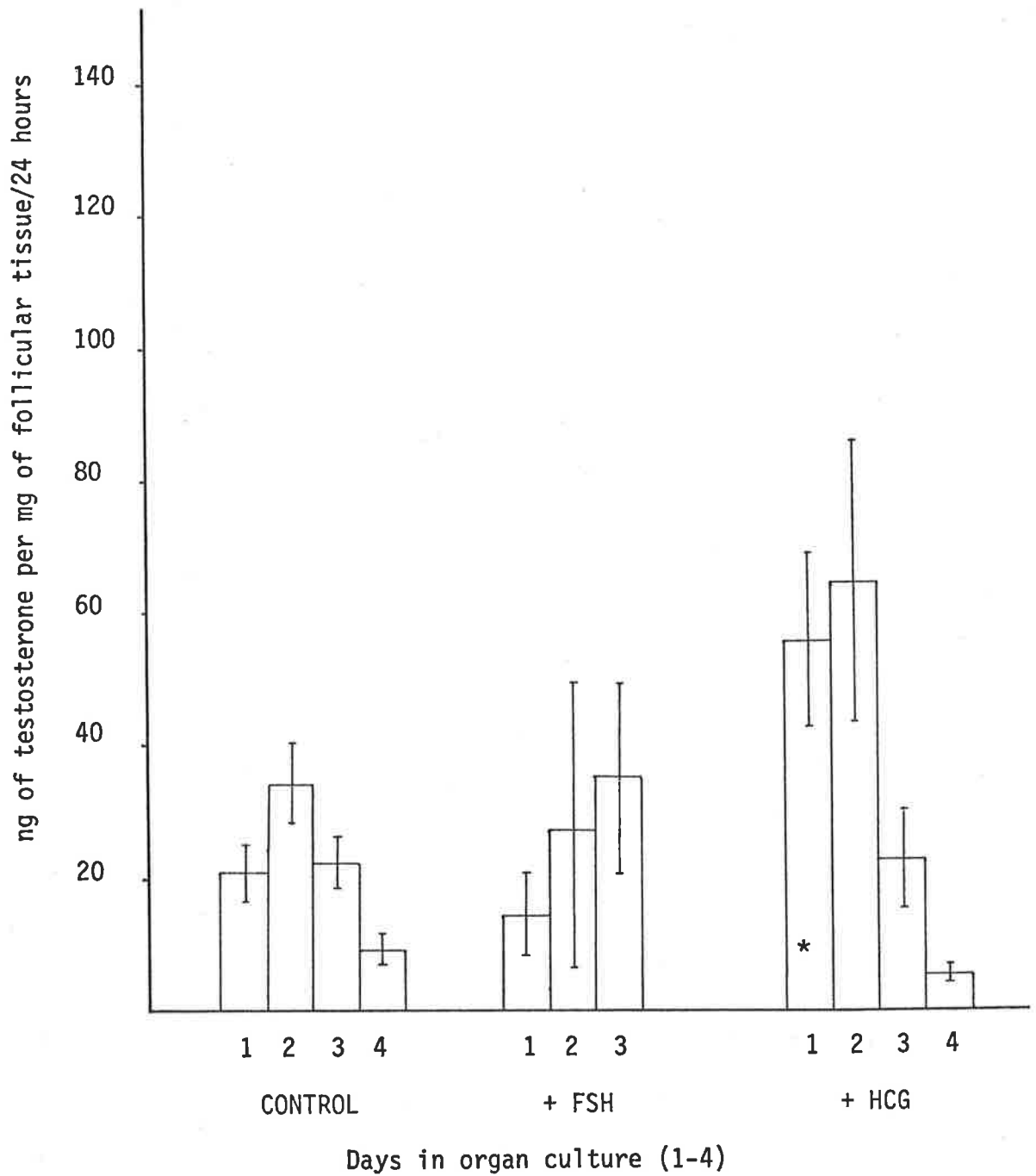
Luteal phase follicles response to gonadotrophins invitro

FIGURE 5.

Comparisons of oestrogen release (ng/mg of follicular tissue per 24 hours) during 4 days in organ culture (mean \pm SEM is shown for each day in organ culture), during incubation:-

- (1) without the addition of a gonadotrophin (n = 7)
- (2) during incubation with FSH (n = 3)
- (3) during incubation with HCG (n = 5)

These follicles were explanted during the luteal phase (days 15-28) of the menstrual cycle.

* Notation of significant differences are as shown in the legend of Figure 1.

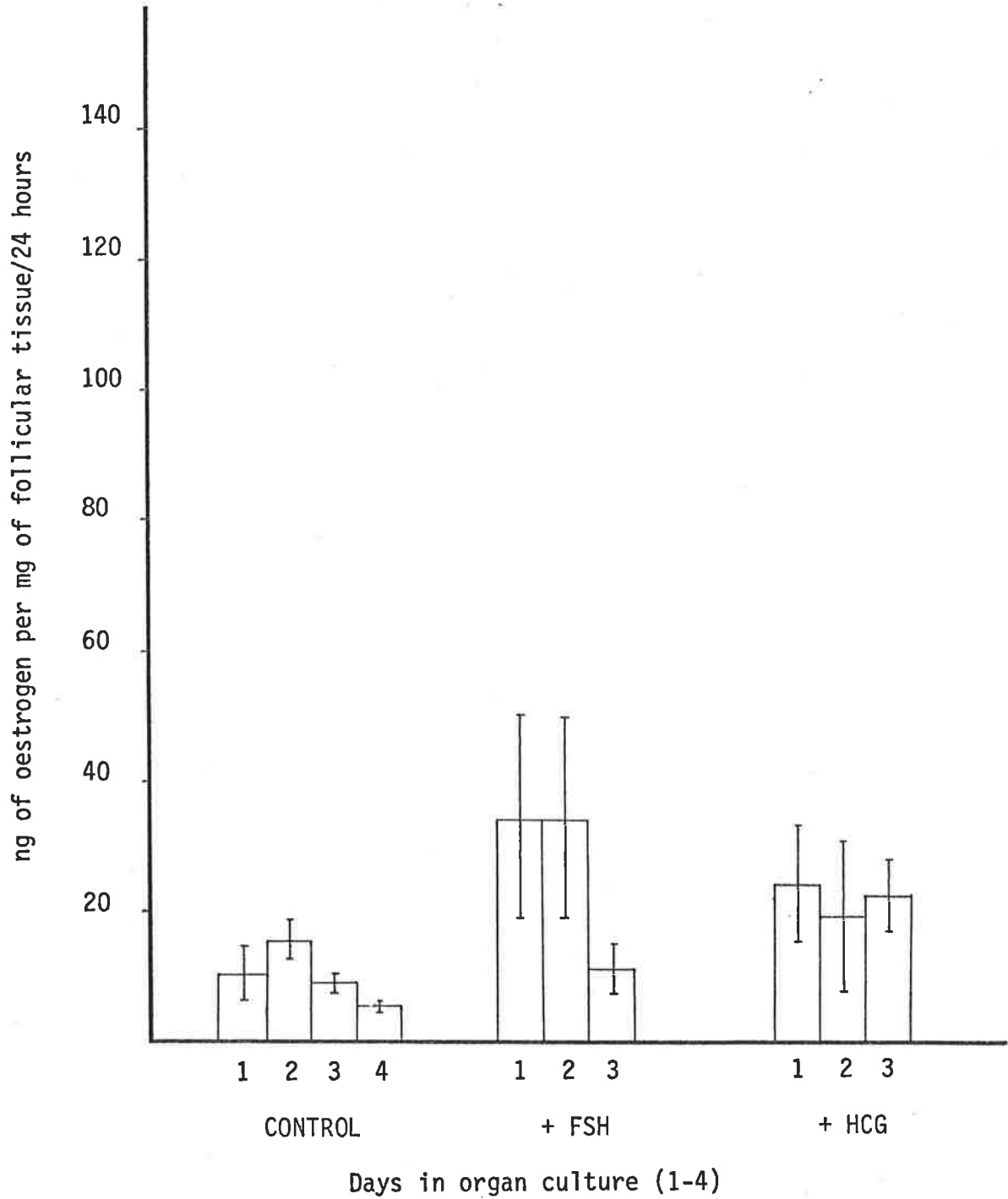
Luteal phase follicles response to gonadotrophins invitro

FIGURE 6.

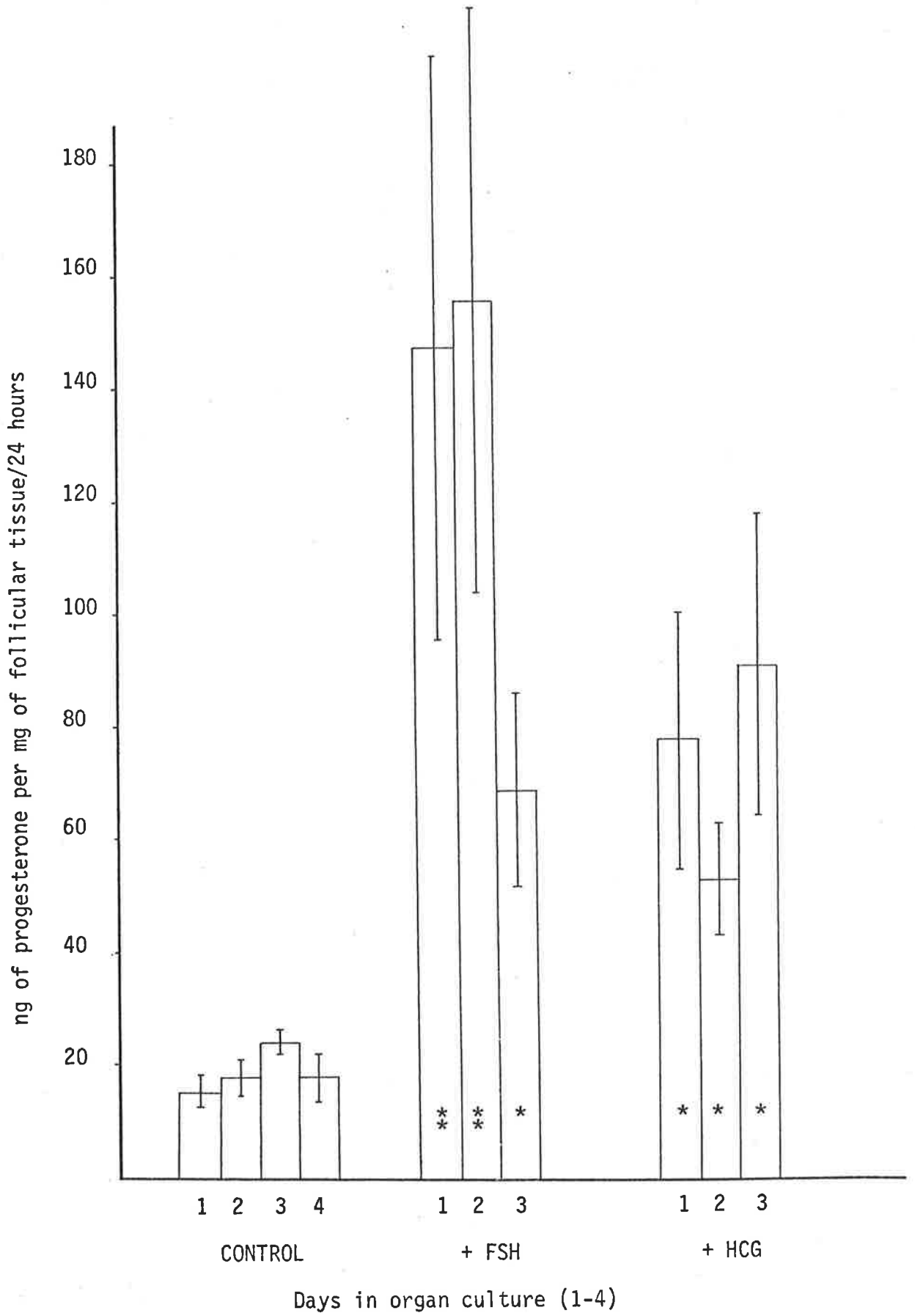
Comparisons of progesterone release (ng/mg of follicular tissue per 24 hours) during 4 days in organ culture (mean \pm SEM is shown for each day in organ culture), during incubation:-

- (1) without the addition of a gonadotrophin (n = 7)
- (2) during incubation with FSH (n = 3)
- (3) during incubation with HCG (n = 5)

These follicles were explanted during the luteal phase (days 15-28) of the menstrual cycle.

* Notation of significant differences are as shown in the legend of Figure 1.

Luteal phase follicles response to gonadotrophins invitro



3.4 DISCUSSION

It has been demonstrated that human Graafian follicles, when cultured under the conditions described, altered their steroid activity during incubation with gonadotrophins. These alterations in steroid activity varied, and were dependant upon the preparation of gonadotrophin used (human pituitary FSH, CSL or chorionic gonadotrophin, LH, Pregnyl, organon) and the time of explantation of the follicle during the reproductive cycle.

The early follicular phase follicles (average diameter of 5.4 mm) were able to increase their output of testosterone by a small amount and their output of progesterone by a large amount during incubation with HCG. This observation indicates that these follicles have functional luteotrophic receptor sites. Evidence that the granulosa cells were the primary source of progesterone release during incubation with HCG was found by observing that the activity of the enzyme 3β -HSD was marked in the granulosa layer of these follicles, (see Chapter 4). These early follicular phase follicles did not tend to undergo spontaneous luteinization during 4 days in organ culture as judged by a low steady progesterone release and minimal 3β -HSD activity in their granulosa cells. A similar observation was noted by Channing (1970), with regard to Rhesus monkey granulosa cells in culture. In this study, follicles less than 1 mm in diameter and also early follicular phase follicles failed to luteinize spontaneously, invitro, but were able to, following incubation with a combination of LH and FSH. It was noted that FSH or LH in isolation failed to bring about luteinization, which tended to substantiate the hypothesis proposed by Greep (1942), that a synergism of FSH and LH was necessary in bringing about normal follicular growth, ovulation and luteinization. Unfortunately incubation of the early follicular phase follicle with FSH was not performed, but would be worthwhile in view of the observation by Weiss and Armstrong (1977), that the granulosa cells from small sheep follicles increase cAMP accumulation during incubation with FSH but not with HCG.

The incubation of late follicular phase follicles with HCG resulted in an increased release of testosterone and a marked supression of oestradiol- 17β release. A fraction of the increased release of testosterone may have been due to a substrate accumulation effect. Hay and Moor (1973), noted that the addition of physiological levels of LH to sheep follicles, invitro, inhibited oestrogen secretion and at the

same time induced luteinization in the granulosa cells and the secretion of progesterone. Furthermore, Seamark et al (1974), measured substrate accumulation after the addition of LH to sheep follicles, invitro. The findings indicated that inactivation of first the aromatase system and then the desmolase system occurred, thereby limiting steroid production via the Δ^5 -hydroxyl pathway in the theca interna. Invivo studies on the cow showed a precipitous drop in circulating oestrogen after the infusion of LH just prior to ovulation (Dobson and Fitzpatrick, 1975). From the results obtained, a similar mechanism appears to operate in the human follicle, explanted during the late follicular phase of the menstrual cycle. These follicles spontaneously released large amounts of progesterone from day 1 invitro, suggesting that they may have already been luteinized invivo. Histochemical localization of 3β -HSD activity was marked in the granulosa cells of recently explanted ovaries during the preovular period (see Chapter 4). Wheeler, Baird, Land and Scaramuzzi (1975) have demonstrated that the ewe secretes progesterone from the ovary during the preovulatory period. Yussman and Taylor (1970), were able to show from observations in women, that the plasma progesterone rose significantly 16 hours prior to ovulation, then plateaued or fell slightly about the time of ovulation. The same workers noted that a surge of LH and a lesser one of FSH occurred about 24 hours prior to ovulation. The effect of gonadotrophins on ovarian function and in turn the effect of the ovarian steroids on peripheral target organs has been demonstrated, (Kerin, Matthews and Svigos, 1976 and Kerin, 1977). It was found that both the cervical mucus score and sperm penetration was maximal on the day of the LH peak. However within 24 hours of the LH peak both the cervical mucus score and the ability of sperm to penetrate the mucus had declined. This observation was indirect evidence that circulating 17α -OH-progesterone and progesterone levels were already high enough just prior to or during ovulation to have an adverse effect on cervical mucus and sperm penetration. The knowledge that the FSH preparation also contained significant quantities of LH, and that this preparation augmented progesterone and oestradiol- 17β release in late follicular phase follicles, whereas HCG did not, lends support to the thesis that both these gonadotrophins are necessary for optimal follicular steroidogenesis.

The observation that early follicular phase follicles released more testosterone than oestrogen in culture per mg of follicular tissue whereas late follicular phase follicles released more oestrogen than androgen indicated that their aromatase activity was more efficient. Furthermore, the observation that incubation of late

follicular phase follicles with FSH significantly increased oestrogen release, tends to support Moon, Dorrington and Armstrong's hypothesis (1975), that FSH stimulates aromatization of androgens to oestrogens.

The observation that human follicles, explanted during the luteal phase, released only small amounts of oestrogen, invitro, supports similar observations made by Baird and Frazer (1975). Furthermore, these follicles failed to increase their oestrogen release during incubation with either FSH or HCG. This finding suggested that their aromatase activity had diminished, as the substrate testosterone was plentiful and its release increased slightly during incubation with HCG. The observation that luteal phase follicles were able to respond to both FSH and LH for increased progesterone production, indicated that the granulosa cells of these follicles remain functional and tended to reflect the activity of the luteinized granulosa cells of a corpus luteum.

Although the smaller post partum follicles (average diameter, 4.2 mm) produced considerable levels of testosterone, oestradiol-17 β and progesterone, spontaneously, invitro, they failed to respond to incubation with FSH apart from a transient increased output of progesterone. The hormonal environment of these follicles, invivo, during the first week following parturition has been studied by Rolland, Lequin, Schellekens & De Jong (1975). They found that plasma levels of prolactin were high (an observation confirmed in this study, see Chapter 6) and plasma FSH almost undetectable at this time. Ovarian refractoriness to exogenous gonadotrophins in the human female has been demonstrated during the early post partum period, in lactating women (Zarate, Canales, Soria, Ruiz and MacGregor, 1972). The exact mechanism for this ovarian refractoriness is uncertain. Reyes, Winter and Faiman (1972), suggested it could be due to an anti gonadotrophin action of prolactin at an ovarian level. However, Varga, Wenner and Del Pozo (1973), believe in an anti gonadotrophic action of prolactin via the hypothalamus. The observations from this study indicated that these ovarian follicles are refractory from a steroidogenic point of view in the presence of gonadotrophins and support the observations of Reyes et al., 1972 and Rolland et al., 1975, that the ovaries are the more refractory component of the hypothalamic-pituitary-ovarian axis following delivery. It has been shown by McNatty et al (1974), that high follicular prolactin levels may be inhibitory to steroidogenesis,

particularly to progesterone production; a finding supported by observations in this thesis (see Chapter 6). This situation does apply during the early post partum period with respect to high levels of circulation prolactin. The lack of a steroid response by post partum follicles during incubation with FSH could also be due to either sparse or nonfunctional FSH receptor sites in these follicles.

3.5 SUMMARY

Human ovarian follicles were able to respond with respect to alterations in steroid activity, invitro, during incubation with gonadotrophins. Incubation of early follicular phase follicles with HCG was associated with a markedly increased progesterone output. Incubation of late follicular phase follicles with HCG was associated with a suppression of oestrogen release and an increased release of testosterone indicating inactivation of the aromatase system. Incubation of late follicular phase follicles with FSH (which also contained LH) was associated with an increased release of all three sex steroids. The luteal phase follicles failed to produce an increased release of oestrogen during incubation with either FSH or LH but were able to produce a small but significant increased release of testosterone during incubation with HCG. This observation indicated that their aromatase activity had diminished. These same luteal follicles were able to respond to both FSH and LH for increased progesterone release, which indicated that their granulosa cells remained functional. Although post partum follicles produced steroids, invitro, they failed to respond to FSH apart from a transient increased output of progesterone. These observations support the concept that the ovaries are a refractory component to gonadotrophic stimulation within the hypothalamic-pituitary-ovarian axis system following delivery. This observation was consistent with the hypothesis that FSH receptor sites were either sparse or non functional in these follicles, or that their invivo, high prolactin - low FSH environment at the time of explantation influenced their steroid activity, invitro.

4. A HISTOCHEMICAL ASSESSMENT OF THE SITE AND ACTIVITY OF THE ENZYME Δ^5 -3 β -HYDROXYSTEROID DEHYDROGENASE (3 β -HSD) IN RELATION TO THE STEROID ACTIVITY OF HUMAN GRAAFIAN FOLLICLES IN ORGAN CULTURE, EXPLANTED THROUGHOUT THE MENSTRUAL CYCLE AND IN THE IMMEDIATE POST PARTUM PERIOD: COMPARISONS BETWEEN FOLLICLES INCUBATED WITH AND WITHOUT GONADOTROPHINS

4.1. Introduction

All the hormonally active steroids in the human ovary are synthesized biologically by pathways involving the oxidation of the Δ^5 -3 β -hydroxyl group to the Δ^4 -3-ketone group. The enzyme system which catalyses this oxidation step is Δ^5 -3 β -HSD. This enzyme has a well defined position in steroid biosynthesis (Samuels, Helmreich, Lasater and Reich, 1951), and catalyses the conversion of pregnenolone to progesterone and of dehydroepiandrosterone to androstenedione.

The microscopic histochemical method for the demonstration of steroid 3 β -ol-dehydrogenase, used in this study was first developed and described by Wattenberg in 1958. Hay and Moor (1975), used this method to examine the distribution of 3 β -HSD activity in the Graafian follicle of the sheep and related it to their oestrogen and progesterone production. They were able to show that high oestrogen secretion by follicles, invitro, was associated with 3 β -HSD activity in the theca interna cells and after the addition of LH to the cultured follicles, oestrogen secretion was inhibited and progesterone secretion enhanced with the appearance of 3 β -HSD activity in the granulosa cells. Friedrich, Breiteneker, Salzer and Holzner (1974), were able to demonstrate the occurrence of progesterone in the follicular fluid of large preovular human Graafian follicles and histochemical evidence of 3 β -HSD activity in the granulosa cells.

A preliminary report has been made correlating invitro steroid release with the histochemical localization of concentration of a reaction product denoting 3 β -HSD activity in the theca interna and granulosa cells of human ovarian follicles explanted during the early follicular, late follicular and luteal phase of the menstrual cycle (Kerin, Seamark and Cox, 1977).

4.2. Theoretical considerations with respect to assessing 3 β -HSD enzyme activity by histochemical methods

4.2.1. Properties of the Tetrazolium salts

The function of the tetrazolium salts (in particular Nitro-Blue

Tetrazolium for this study) in dehydrogenase histochemistry is to act as an acceptor of electrons from the oxidized substrate or, more usually, from some intermediate electron carrier. These electrons, if not intercepted, would normally pass by way of a succession of carriers to molecular oxygen.

The dehydrogenase is the first member of a chain of enzymes which transfer hydrogen atoms or electrons from the substrate to molecular oxygen. In histochemical practice the principle aim is to channel the flow of electrons into the tetrazolium trap, so that the maximum amount of formazan is produced. Nitro-BT picks up most electrons at the level of cytochrome b, or from the flavoprotein-cytochrome b complex to form purple formazan products (Pearse, 1968).

The ideal tetrazolium salt employed should have:-

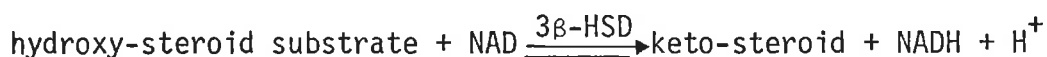
- (1) an adequate oxidizing capacity or redox potential,
- (2) a sufficiently small molecular size for the diffusion constant to be high enough to allow rapid penetration of tissues in order to produce patterns of formazan deposition which indicate discrete intracellular localization of dehydrogenase activity,
- (3) a low substantivity factor, which means a low polarity so that it has a low affinity to bind to tissue protein. Significant binding may give a nonspecific positive reaction,
- (4) a resistance to formazan production in or on lipid droplets which may give a misleading positive reaction. Therefore in this procedure the lipid was extracted from the follicles by treatment with acetone (see Methods),
- (5) a low tissue toxicity and enzyme inhibition factor,
- (6) acceptable stability when exposed to light.

Nitro Blue tetrazolium (Nitro-BT) satisfies numbers 1, 2, 4, 5 & 6 of these criteria, but does tend to bind to tissue protein (3), a factor that may account for the occasional nonspecific positive reaction in tissue preparations. A more complete account of these considerations is given by Pearse, (1968).

4.2.2. The Role of Coenzymes (see Diagram 1, p 116)

The specific coenzymes act as carriers in the passage of electrons and hydrogen from the original substrate (e.g., pregnenolone, 17α -OH-pregnenolone and dehydroepiandrosterone) to molecular oxygen. The

coenzyme concerned in this study was nicotinamide-adenine dinucleotide (NAD). This coenzyme is readily and reversibly reduced by the dehydrogenase and in turn acts as a substrate for further enzyme systems.



4.2.3. The Role of Diaphorases

The diaphorases are a series of enzymes which catalyse the oxidation of reduced coenzymes. The diaphorase relevant to this study was the one responsible for the oxidation of reduced NADH (or coenzyme I) to NAD, with the consequent reduction of cytochrome c, thereby taking the electron transport system of the cell a step further.

4.2.4. Reasons for the addition of Coenzymes

Despite the apparent preservation of mitochondrial morphology achieved by the use of protective media, the freezing and thawing of the normal cold microtome (cryostat) procedure results in damage to the mitochondrial membrane and the release of soluble co-factors (i.e. NAD). The endogenous levels of coenzymes are usually too low to allow histochemical dehydrogenase reactions (coenzyme-dependent) to take place at all and therefore exogenous NAD must be added (see Methods). For this histochemical study it was found that the optimal reduction of tetrazolium salt (Nitro-BT) occurred when the coenzyme (NAD) was present in a similar concentration as the substrate (DHEA).

4.2.5. Substrate and Formazan Specificity

Wattenberg (1958), tested several steroids for substrate activity in the reaction mixture used for the demonstration of steroid-3 β -ol-dehydrogenase. He found that only dehydroepiandrosterone and pregnenolone, which are established substrates for this enzyme, resulted in a positive reaction. One substrate he did not test was 17 α -hydroxy-pregnenolone. In this study it was found that this substrate in ovarian follicular tissue was acted upon by 3 β -ol-dehydrogenase, (see Methods & Results). Wattenberg was also able to show that the diffusion artifact was extremely minimal, that the formazan pattern observed in this histochemical method was specific for 3 β -ol-dehydrogenase as distinct from

other NAD linked dehydrogenases and also that where there was histochemical evidence of 3β -ol-dehydrogenase activity there was always evidence of NAD dependent diaphorase activity.

4.2.6. Histochemical localization of 3β -HSD activity

The conversion of the Δ^5 -hydroxy steroids to Δ^4 -keto steroids requires the presence of the NAD dependent enzyme 3β -HSD, which catalyses the removal of the hydrogen molecule of the 3β -hydroxyl group and its subsequent conversion to a ketone group. The free hydrogen ion is picked up by NAD. The enzyme NAD diaphorase catalyses the oxidation of NADH with the consequent reduction of the cytochrome complexes. In the presence of colourless water soluble Nitro-BT (which is an avid hydrogen ion acceptor) the cytochromes are oxidized and Nitro-BT reduced to a formazan compound which is a deep purple water insoluble tissue bound molecule. This insoluble coloured complex denotes the site of 3β -HSD activity. See Diagram 1. (p 122)

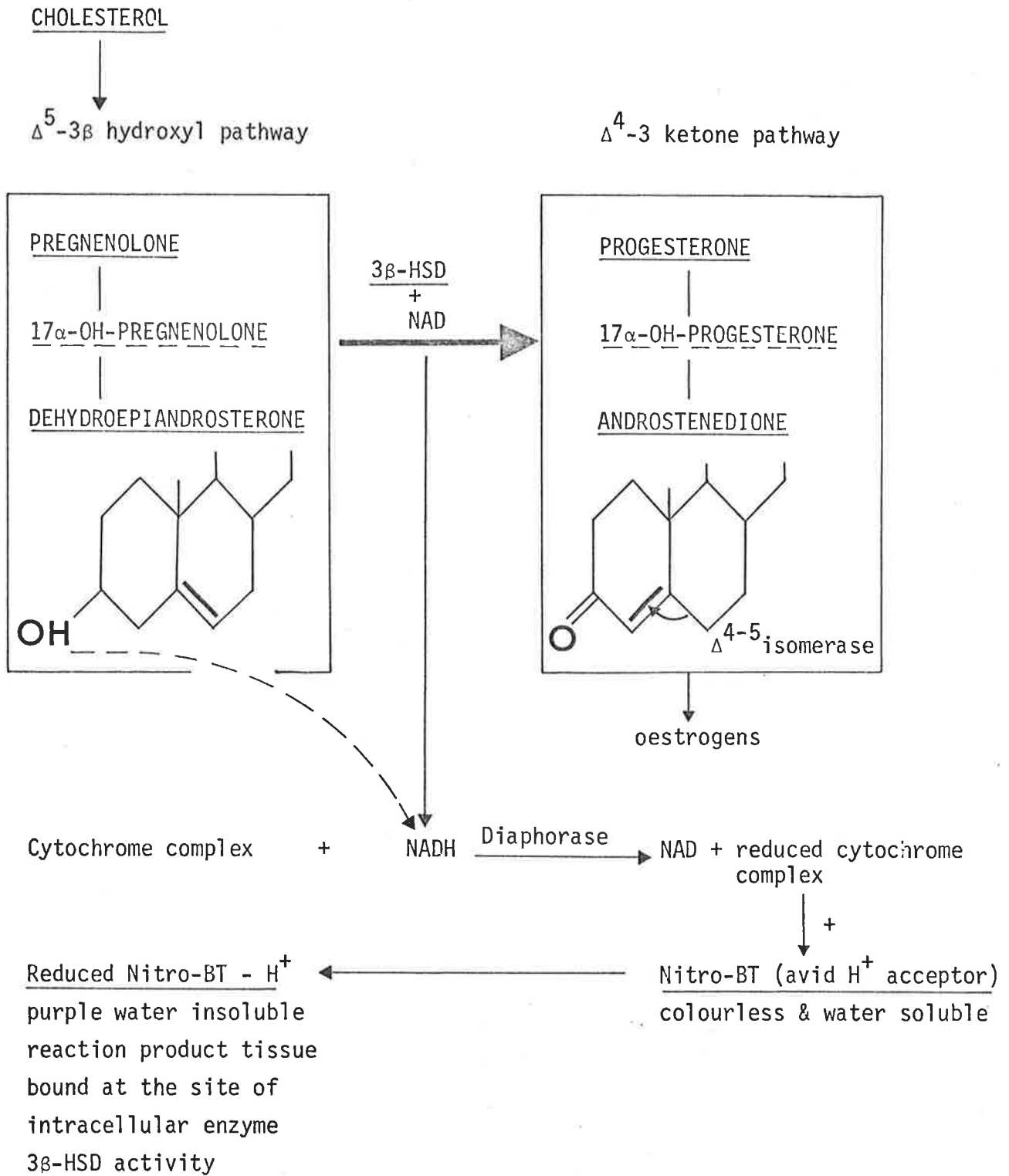
4.3. Materials & Methodology

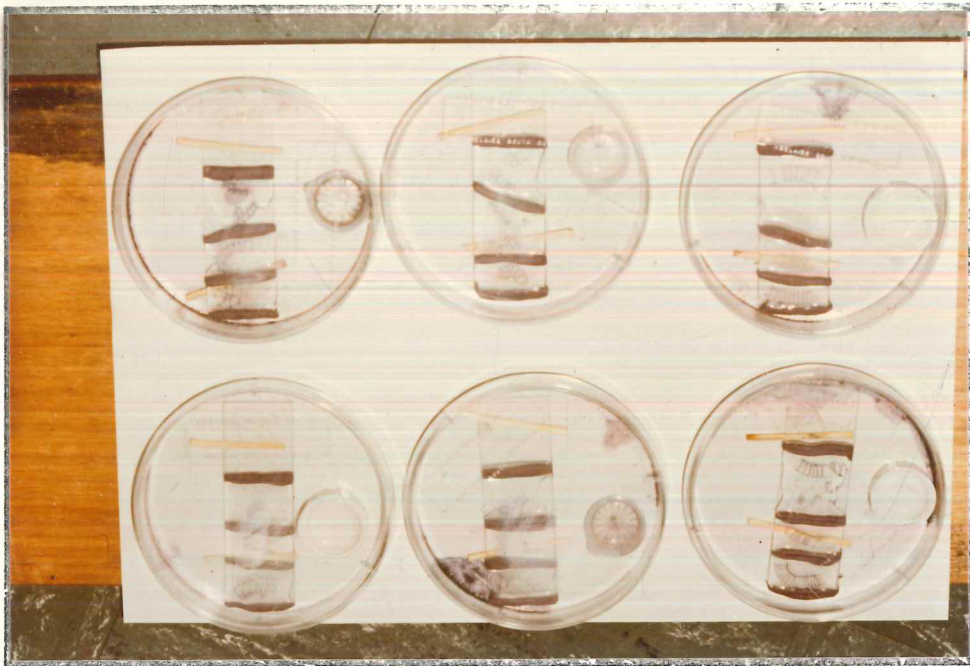
4.3.1. Materials

1. Cryo-Cut Microtome (American Optical Corporation).
2. Tissue-Tek II O.C.T. Compound (Miles Laboratories Inc., Illinois, U.S.A.).

3. <u>Incubating medium consisted of:-</u>		<u>Volume</u>
(1) Nicotinamide adenine dinucleotide (NAD)	6.6 mg/ml	0.1 ml
(2) $MgCl_2$	4.8 mg/ml	0.1 ml
(3) Nitro-Blue Tetrazolium (DMF-dimethyl formamide)	2 mg/ml in DMF	0.1 ml
(4) Dehydroepiandrosterone (DHA)	5 mg/ml in DMF	0.1 ml
(5) Tris buffer (pH 8.3, 0.2M)		0.6 ml
4. Acetone (cold, - 20°C)		
5. Neutral formal saline		
6. Ethanol, 20%		
7. Methyl green, 2% or 0.2% neutral red chloride		
8. Glycerine jelly		
9. Incubator		

Diagram 1. Histochemical mechanism for localization & quantitation of Δ^5 - 3β hydroxysteroid dehydrogenase enzyme activity

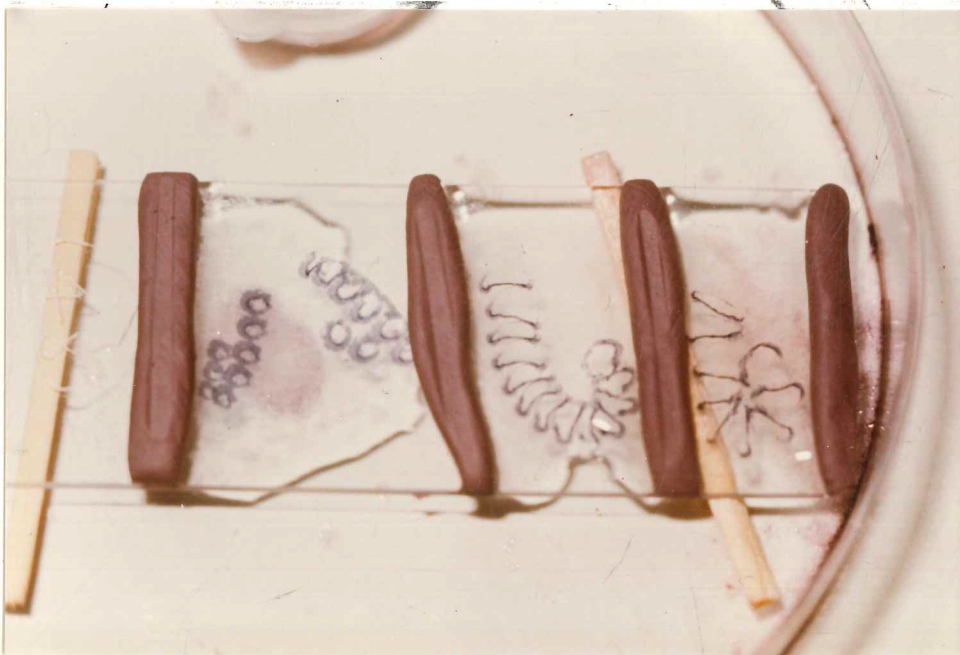




Photograph 8. (x1/3)

Pre-incubation

A series of six follicles which have been serial sectioned are in separate dishes of histochemical incubation medium for the estimation of 3β -HSD activity.



Photograph 9. (x1½)

Post-incubation

Serial slices through a single follicle from its top to the base which was resting on the steel grid. Follicular slices are divided by plasticine strips to localize the incubation fluid over the follicular sections and also to allow for simultaneous incubation of the tissue from one follicle with different substrates for comparative studies.

4.3.2. Methodology

4.3.2. (1) Preparation of 'Tris' Buffer (pH 8.3, 0.2M)

The procedure used was taken from Cullings textbook (1974), 3rd Edition, Hand book of Histopathological & Histochemical Techniques. Chapter 8 - Preparation of Stains. p 174.

Solutions required:

1. 0.2 M tris (hydroxymethyl) aminoethane
2. 0.1 N hydrochloric acid (HCl)

In order to make up a tris buffer solution of pH 8.3, 25 ml of 0.2 M tris + 20 ml of 0.1 N HCC made up to 100 ml with distilled water was used.

4.3.2. (2) Method for Histochemical Determination of 3 β -Hydroxysteroid Dehydrogenase Activity. (Modification of the description by Pearse, 1968)

This histochemical method was developed by Wattenberg (1958), and used by Hay and Moor (1975). Immediately after the Graafian follicles were removed from the culture incubation medium they were imbedded in Tissue-Tek and rapidly frozen onto prefrozen microtome 'chucks' to -18°C . Serial frozen sections, 12 μm thick were obtained using a Cryo-Cut microtome. A technique was developed whereby serial slices of the whole follicle, including in many cases the ovum and surrounding granulosa cells could be obtained and incubated on the one slide. In this way a semi quantitative assessment could be made of the intensity and pattern of the formazan reaction product in the theca interna, the granulosa layer and in the granulosa cells surrounding the ovum, (see pre and post incubation photos).

In all cases, unless specifically stated, the steroid substrate used was dehydroepiandrosterone (DHA) in dimethyl formamide (DMF). The co-factor used was nicotinamide adenine dinucleotide (NAD). For cellular contrast the nuclei were counter stained with either 2% methyl green or 0.2% neutral red chloride, in order to highlight the purple formazan reaction product.

4.3.2. (3) Methodological Steps

1. Allow the fresh, frozen cryostat section of the follicle to dry onto a microscope slide for 2 minutes.

2. Remove lipids by immersing the slides into cold acetone (-20°C) for 20 minutes.
3. Dry sections in air for 5-10 minutes.
4. Add the freshly made up incubation medium to each slide containing slices of follicles and enclose in a humidified Petri dish.
5. Incubate at 37°C for 30 minutes.
6. Rinse slides briefly and gently in distilled water.
7. Fix in neutral formal saline for 30 minutes.
8. Rinse slides in distilled water and treat for 2 minutes with 20% ethanol.
9. Rinse slides again in distilled water and counterstain nuclei in 2% methyl green or 0.2% neutral red chloride. Wash off excess counterstain after 30 seconds with water.
10. Mount with a coverslip using glycerine jelly.

4.3.3. Histochemical Controls

4.3.3. (1) Omission of the substrate

During each histochemical experiment a representative sample of tissue was incubated in media under identical conditions with one exception; the omission of the substrate (DHA). In all cases there was either no formazan reaction product or an amount of minimal intensity (histochemical score 0-1).

4.3.3. (2) Omission of the coenzyme

The omission of coenzyme (NAD) in the incubation media was followed by an absence of a formazan reaction product in control sections. This observation supports Wattenberg's conclusions (1958), see 4.2.4.

4.3.3. (3) Heat denaturation

Slices of ovarian follicle heated to 60°C for sixty seconds and then processed for histochemical evaluation of 3β -HSD showed no evidence of formazan formation. This procedure indicates that enzymic denaturation had occurred as suggested by Hay and Deane (1966).

4.3.3. (4) Exogenous steroid suppression of enzyme activity

Ovarian biopsies were performed on women who were currently on a combined oestrogen-progesterone contraceptive pill preparation for a period of at least three months. There were no visible follicles present, slices of these ovaries were inactive steroidogenically in culture and they were also negative for formazan deposition following histochemical

analysis for 3β -HSD. In other words there was no evidence of nonspecific or false positive formazan reaction product produced, for which the term "nothing dehydrogenases" has been used (Hay and Deane, 1966; Pearse, 1968).

4.3.4. A histochemical score of 3β -HSD steroid activity

An attempt to semiquantitate the activity of the enzyme 3β -HSD was performed at the sites of formazan reaction product formation.

A histochemical score of:-

- 0 = no formazan reaction product
- 0-1 = minimal formazan reaction product
- 1-2 = moderate formazan reaction product
- 2-4 = marked formazan reaction product

The higher histochemical score implicated that enzyme activity was correspondingly high at these sites of formazan deposition. Evidence to support this supposition was found when the histochemical scores were correlated with the steroid activity of the repective follicles, (see 4.9.).

4.3.5. Assessment of Diaphorase Activity

Sections of human ovarian follicles were processed histochemically for the detection of diaphorase activity as described by Wattengerg (1958) and Hay and Deane (1966), for localization of its presence in testicular tissues.

The only alteration in the histochemical methodology was to omit NAD from the incubation medium and add reduced NAD (NADH) in a concentration of 1.5 mg/ml, which acted as the substrate. This procedure demonstrated the presence of NAD-tetrazolium reductase (diaphorase) in the follicular tissues in both the theca and granulosa cells (see diagram 1 for its site of action). This reaction must be positive if NAD dependent enzymes (such as 3β -HSD) are to be visualized. Since diaphorase activity could be demonstrated in all the tissues which were also active for 3β -HSD it could be said that 3β -HSD activity had not been demonstrated in cells lacking diaphorase activity. If there was evidence of 3β -HSD activity in tissues where diaphorase activity was absent, then this method's credibility as an exclusive indicator of 3β -HSD activity would have been in question.

4.4. Preliminary evaluation of steroid activity in relation to the site of 3 β -HSD activity in sheep follicles

4.4.1. Incubation of sheep follicles with and without HCG

Correlations between the steroid release and 3 β -HSD activity in the theca and granulosa layers of incubated sheep follicles were made. The dissection, incubation and histochemical procedures were identical to the ones outlined for human follicles.

Four sheep follicles, ranging from 4 to 7 mm in diameter were incubated for 4 days in gonadotrophin free media and designated as the control follicles. A series of five sheep follicles of similar size to the control follicles were incubated in media which contained 20 iu/ml of HCG.

Results

Table 1

Days in organ culture	Incubation of <u>unstimulated follicles</u> (n = 4) in organ culture. Steroid release expressed as ng of steroid (mean \pm SEM) per mg of follicular tissue/24 hrs		
	Testosterone	Oestrogen	Progesterone
1	18 \pm 2	44 \pm 7	2 \pm 0.3
2	25 \pm 4	50 \pm 8	4 \pm 1.0
3	21 \pm 3	42 \pm 8	3 \pm 0.3
4	17 \pm 2	33 \pm 6	5 \pm 1.4

Histochemical localization of enzyme 3 β -HSD activity, following incubation of these control follicles in organ culture.

Table 2

	Histochemical Score	
	Theca	Granulosa
Follicle 1	++ = 2	+ = 1
" 2	+++ = 3	+ = 1
" 3	+++ = 3	- = 0
" 4	+++ = 3	+ = 1

Table 3

Days in organ culture	Incubation of follicles with HCG (20 iu/ml), n = 5 Steroid release expressed as ng of steroid (mean \pm SEM) per mg of follicular tissue per 24 hours		
	Testosterone	Oestrogen	Progesterone
1	17 \pm 1.7	48 \pm 8	12 \pm 4
2	6 \pm 0.7	23 \pm 3	123 \pm 12
3	0.9 \pm 0.1	7 \pm 0.6	109 \pm 14
4	0.6 \pm 0.1	1.7 \pm 0.2	98 \pm 23

Histochemical localization of 3β -HSD activity, following incubation of these 5 follicles with HCG.

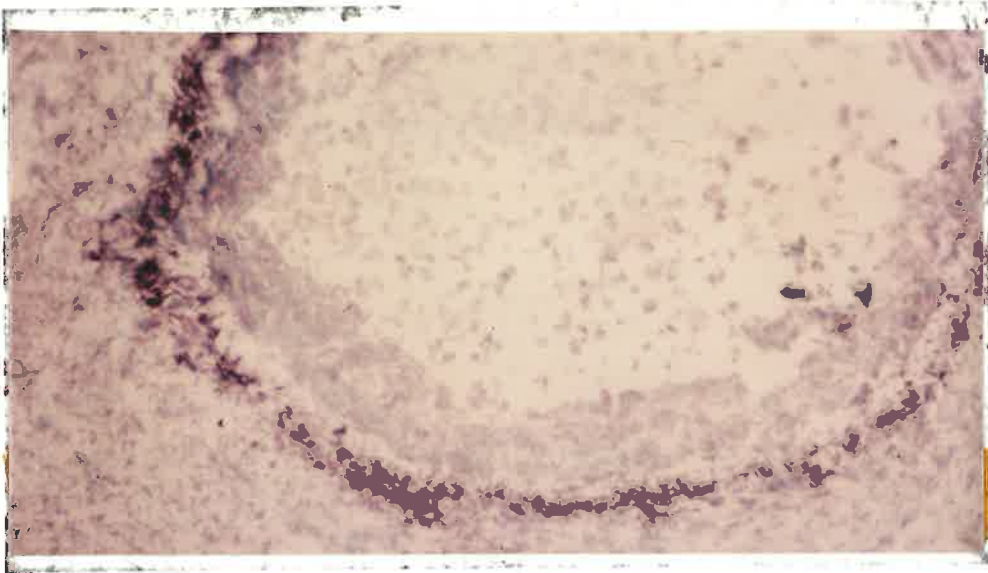
Table 4

	Histochemical Score	
	Theca	Granulosa
Follicle 1	+ = 1	+++ = 3
" 2	+ = 1	+++ = 3
" 3	- = 0	+++ = 3
" 4	++ = 2	+++ = 3
" 5	+ = 1	+++ = 3

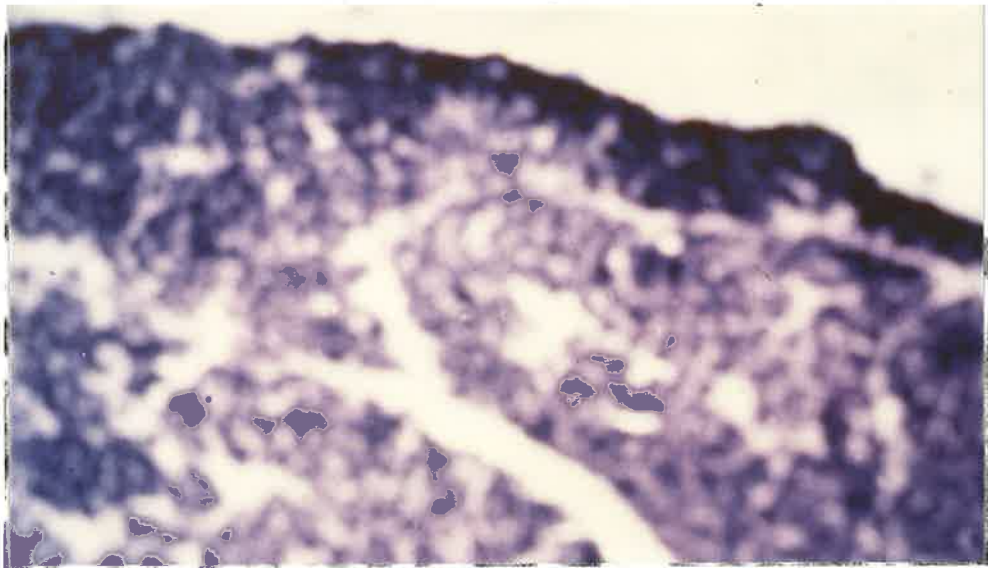
Discussion

This preliminary experiment using 9 sheep follicles in organ culture, supported the findings of Hay and Moor (1975). The control follicles released steady levels of testosterone and oestrogen but very low levels of progesterone. Most of the 3β -HSD enzyme activity was confined to the theca interna cells (see Table 1 and 2).

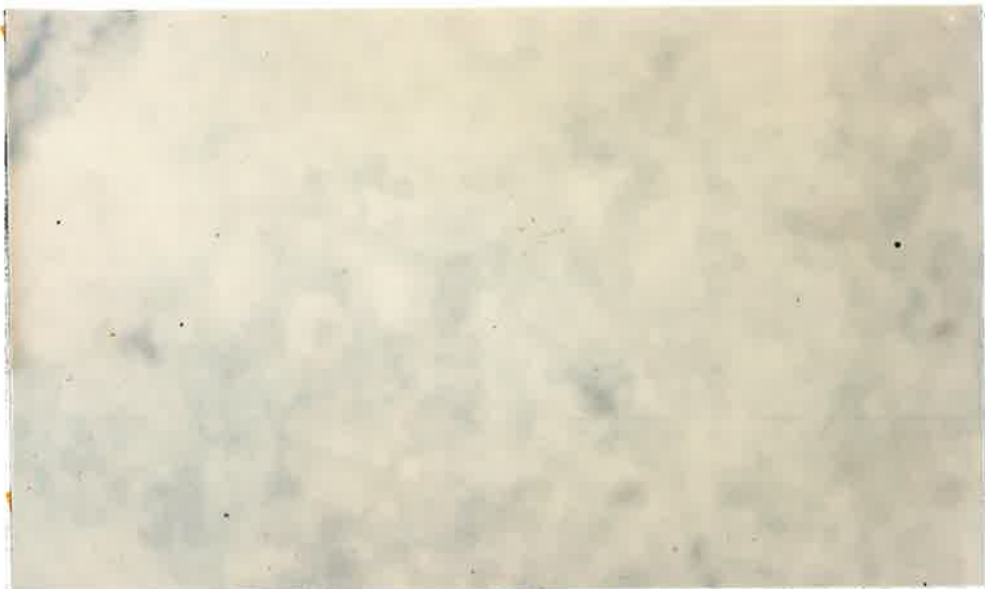
The addition of HCG to the culture medium was associated with a suppression of oestrogen and testosterone release and a stimulation of progesterone release. This effect was evident within 48 hours following incubation with HCG. Histochemical localization for the activity of 3β -HSD demonstrated most of the formazan reaction product in the granulosa cells, at the time when these follicles were releasing progesterone predominantly in culture (see Table 3 and 4).



Photomicrograph 10. (x30)
Ovarian follicle - 1000 rad.



Photomicrograph 11. (x30)
Corpus luteum - 1000 rad.



Photomicrograph 12. (x30)

An identical series of experiments were carried out using higher concentrations of HCG in the culture media as follows, 20 iu/ml, 50 iu/ml and 100 iu/ml. There was no significant difference in the steroids released or the histochemical activity of 3β -HSD, using these levels of HCG when compared to using 10 iu/ml in the medium.

4.4.2. The effect of irradiation on the steroid and 3β -HSD enzyme activity of sheep ovaries

This series of experiments were done in collaboration with P.O. Janson, O.M. Petrucco and T. Weiss. The author performed the histochemical and histological studies on these ovaries.

Sheep were run with vasectomized marker rams so that oestrus could be determined. Laparotomies were done on these sheep during the mid luteal phase of the oestrus cycle. Both ovaries were exposed to varied doses of megavoltage irradiation. The doses employed were either 750, 1,000 or 1,500 rad given as a single total dose. Particular note was made of the presence or absence of a corpus luteum in the ovary. Each ovarian vein was cannulated and each cannula brought to the skin surface in order to aspirate ovarian venous blood post operatively. Ovarian venous blood was aspirated on the seventh post operative day for the estimations of testosterone, oestrogen and progesterone. Ten days following the single dose of ovarian irradiation, the sheep were sacrificed and the ovaries examined both histochemically and histologically.

Photographic recordings of the effect irradiation had on ovarian 3β -HSD activity

1. Exposure to 750 rad

3β -HSD activity was still normally evident in both the theca and granulosa layer and was very similar to the appearance seen in photomicrograph 18.

2. Exposure to 1000 rad

Photomicrograph 10.

The granulosa layer is visibly damaged and many cells have become attached. There is not significant 3β -HSD enzyme activity in the granulosa layer of this luteal phase follicle (in contrast to control luteal phase follicles). The formazan reaction product in the theca interna layer is patchy and there is evidence of cellular disorganization.

Photomicrograph 11.

The formazan reaction product indicating 3 β -HSD activity in the corpus luteum following 1000 rad was not significantly impaired when compared to a control corpus luteum.

3. Exposure to 1500 rad

Photomicrograph 12.

This most dismal photograph (which is correctly focused), demonstrates extensive ovarian tissue damage (from the lack of intra-ovarian detail). Although it is not readily apparent this is a section through a corpus luteum which is totally negative for formazan reaction product, indicating total absence of 3 β -HSD enzyme activity.

Results

The results are confined to the ovary containing the corpus luteum.

Parameter assessed	Control n = 3	750 rad n = 3	1000 rad n = 3	1500 rad n = 3
Ovarian venous blood steroid levels ($\bar{X} \pm \text{SEM}$)				
Progesterone (ng/ml)	209 \pm 35	183 \pm 29	155 \pm 27	4.7 \pm 1.2
Oestradiol-17 β "	0.092	0.07	0.08	-
Testosterone "	0.07	0.05	0.06	-
Macroscopic appearance of ovary	Normal	Normal	Normal	Small; no follicles, small C.L.
Histological assessment	Normal	Normal	C.L. normal follicle degenerative changes	C.L. & follicular destruction
3 β -HSD activity				
Corpus luteum	Marked	Marked *	Moderate	Absent
follicle	theca	Marked	Minimal	Absent
	granulosa	Minimal	Absent	Absent

Discussion

This experiment demonstrated that ovarian follicles were more susceptible to the irradiation effects of 1000 rad than the corpus luteum from both a histological and histochemical point of view. However both the follicle and corpus luteum were destroyed by 1500 rad. The experimental design was not sensitive enough to assess follicular changes in steroid activity. Since the sheep corpus luteum almost exclusively produces progesterone it can be concluded that progesterone secretion by the sheep corpus luteum, *invivo*, does not appear to be significantly suppressed until exposed to an irradiation dose of greater than 1000 rad. A significant drop in progesterone secretion followed exposure of the ovary containing the corpus luteum to an irradiation dose of 1500 rad. This experimental procedure may offer a method of selective destruction of intraovarian compartments by controlled doses of irradiation. In this way it may be possible to study selective corpus luteum steroid activity within an ovary, following obliteration of ovarian follicular function by means of a regulated dose of megavoltage irradiation.

This experiment on sheep ovaries was included because it clearly demonstrated that the steroid activity of the corpus luteum in terms of progesterone secretion was closely correlated with the histochemical localization of 3β -HSD activity following ovarian exposure to increased doses of megavoltage irradiation.

Conclusion

These two preliminary studies on cultured sheep follicles illustrated that meaningful correlations could be made between steroid and histochemical investigations *invitro*, and served as a useful prototype on which to design similar experiments using human follicles.

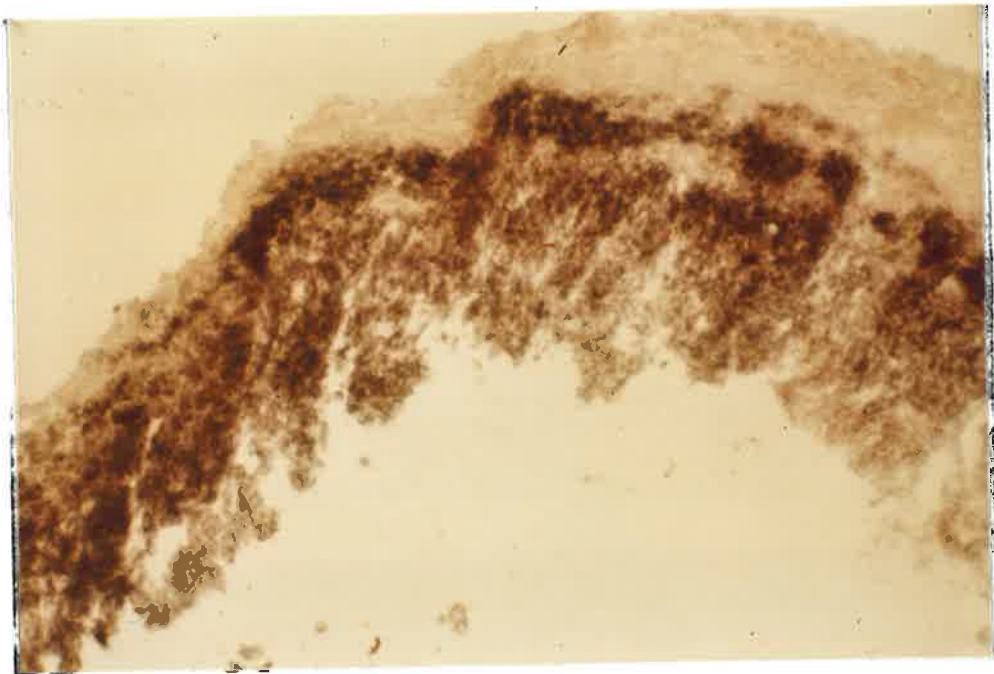
* In light of Wiener's work (1971 - see discussion) it was not possible to say if the 3β -HSD activity within the corpus luteum was significantly more marked after exposure to 700 rad of megavoltage irradiation therapy. The initial impression from this preliminary work was that 3β -HSD activity was at least as active and possibly more active than the activity observed in control corpora lutea. The point of interest being that Wiener postulated that x-irradiation may destroy a specific inhibitor of 3β -HSD activity.



Photomicrograph 13. (x100)

Early follicular phase follicle

Localization of marked 3β -HSD activity is confined to theca cell layer. (Counter stain - 2% methyl green).



Photomicrograph 14. (x100)

Late follicular phase follicle

Moderate localization of 3β -HSD activity is evident in both the theca and granulosa cell layers. (Counter stain - 0.2% neutral red chloride).

4.5. RESULTS

4.5.1. Assessment of 3 β -HSD activity in unstimulated follicles invitro. Comparisons between follicles explanted during the early follicular, late follicular and luteal phase of the menstrual cycle

Histochemical examination of follicles after culture revealed moderate to marked 3 β -HSD activity in the theca cells of follicles explanted during the early follicular, late follicular and luteal phase of the menstrual cycle. The degree of activity of the enzyme was not significantly different in the theca interna layer at any stage of the menstrual cycle in follicles greater than 3 mm in diameter. In the granulosa layer, however, only minimal 3 β -HSD activity was detectable in the early follicular phase follicles, but marked to moderate activity was present in late follicular phase and luteal phase follicles. See photograph 13 and 14 and Figure 1. (The degree of 3 β -HSD enzyme activity in the granulosa layer of early follicular phase follicles was significantly less when compared to late follicular and luteal phase follicles, $p < 0.05$).

4.5.2. Assessment of 3 β -HSD activity following the incubation of early and late follicular phase follicles with gonadotrophins

Histochemical examination of follicles explanted during the early follicular phase (days 1-7), following incubation with HCG showed a significant increased activity of 3 β -HSD activity in the granulosa layer when compared to unstimulated early follicular phase follicles ($p < 0.1$). There was no significant change of 3 β -HSD activity in the theca layer of these follicles following incubation with HCG.

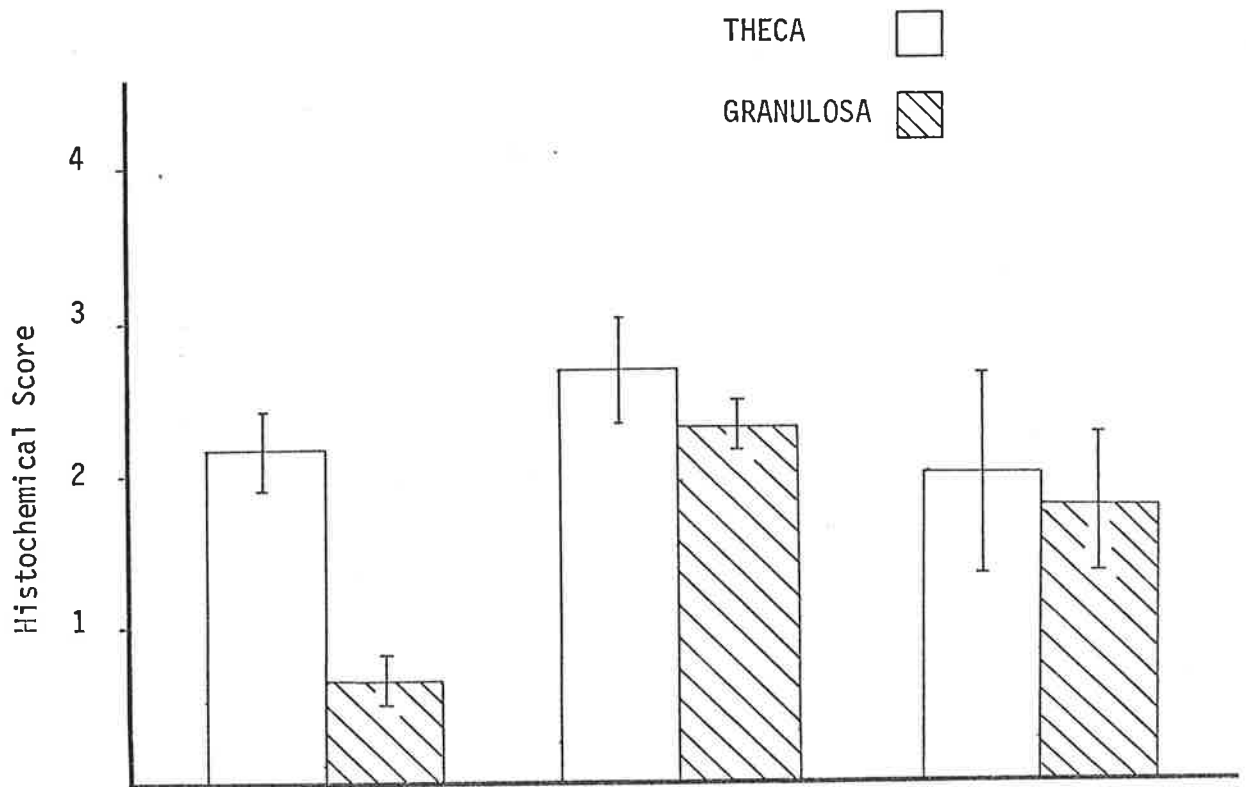
Incubation of follicles explanted during the late follicular phase (days 8-14) with either FSH or HCG failed to show significant differences in 3 β -HSD activity in the theca and granulosa layers when compared to the unstimulated late follicular phase follicles, see Figure 2.

FIGURE 1.

Histochemical localization and activity of 3β -HSD in cultured ovarian follicles (means \pm SEM) following organ culture from follicles explanted during the early follicular phase (n = 5), late follicular phase (n = 6) and luteal phase (n = 6). The steroid output of these follicles during the period in culture preceding histochemical evaluation is shown in Table 1 of Chapter 2.

A histochemical score of 0 = no activity, 0-1 = minimal activity, 1-2 = moderate activity, 2-4 = marked activity of the enzyme 3β -HSD.

Asterisks indicate the significant differences of means for 3β -HSD activity compared with that in late follicular phase follicles, where * $p < 0.1$ and ** $p < 0.05$.

4.5.1. 3β -HSD activity in unstimulated follicles invitro

	Early follicular	Late follicular	Luteal phase
THECA 3β -HSD activity (1→4)	2.2 ± 0.3 (marked)	2.7 ± 0.4 (marked)	2.0 ± 0.7 (moderate)
GRANULOSA 3β -HSD activity (1→4)	** 0.7 ± 0.2 (minimal)	2.3 ± 0.2 (marked)	1.8 ± 0.5 (moderate)
Average diameter of follicles (mm)	* 5.4 ± 0.5	8.5 ± 0.8	* 5.5 ± 0.5

4.5.3. Assessment of 3 β -HSD activity following the incubation of luteal phase follicles with gonadotrophins

Histochemical examination of follicles explanted during the luteal phase (days 15-28) following incubation with FSH was not associated with a significant alteration of 3 β -HSD activity in either the theca or granulosa layers when compared to unstimulated control luteal phase follicles. However, incubation of luteal phase follicles with HCG was associated with a significant increased activity of the enzyme 3 β -HSD in both the theca and granulosa layers when compared to unstimulated luteal phase follicles ($p < 0.01$ for both the theca and granulosa enzyme activity differences).

See Figure 3.

4.5.4. Assessment of 3 β -HSD activity following the incubation of post partum follicles with FSH

Histochemical examination of follicles explanted during the early post partum period (days 2-5 after parturition), following incubation with FSH was not associated with a significant alteration of 3 β -HSD activity in either the theca or granulosa layers when compared to unstimulated post partum follicles.

See Figure 4.

FIGURE 2.

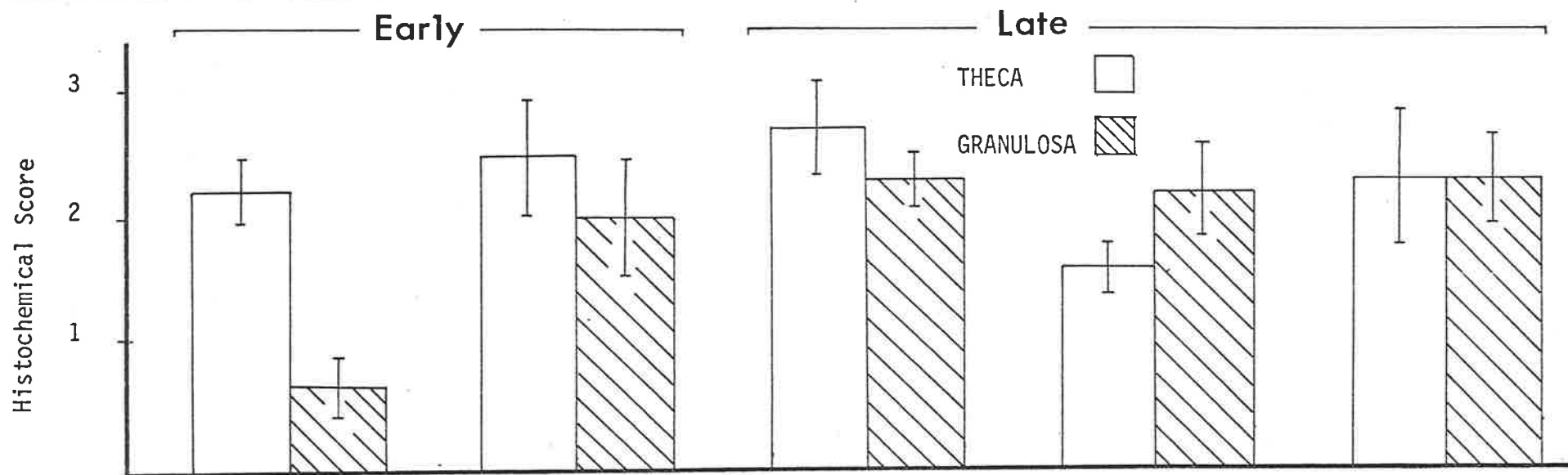
Histochemical localization and activity of 3β -HSD in cultured ovarian follicles (means \pm SEM) which were explanted during the early follicular phase (days 1-7) following incubation:-

- (1) without the addition of a gonadotrophin (n = 5)
- (2) during incubation with HCG (n = 4)

and of follicles explanted during the late follicular phase (days 8-14) following incubation:-

- (1) without the addition of a gonadotrophin (n = 6)
- (2) during incubation with FSH (n = 5)
- (3) during incubation with HCG (n = 4)

Asterisks indicate the significant differences of means for 3β -HSD activity following incubation with gonadotrophins compared to control follicles, where * $p < 0.1$ and ** $p < 0.05$.



	Control	+ HCG (20 iu/ml of medium)	Control	+ FSH (20 iu/ml of medium)	+ HCG (20 iu/ml of medium)
	Early follicular phase	Early follicular phase	Late follicular phase	Late follicular phase	Late follicular phase
THECA 3β-HSD activity (1→4)	2.2 ± 0.3 (marked)	2.5 ± 0.5 (marked)	2.7 ± 0.4 (marked)	1.6 ± 0.2 (moderate)	2.3 ± 0.6 (marked)
GRANULOSA 3β-HSD activity (1→4)	0.7 ± 0.2 (minimal)	2.0 ± 0.5 * (marked)	2.3 ± 0.2 (marked)	2.2 ± 0.4 (marked)	2.3 ± 0.3 (marked)
Average diameter of follicles (mm)	5.4 ± 0.5	4.6 ± 0.5	0.5 ± 0.8	7.1 ± 1.1	6.1 ± 0.4

FIGURE 3.

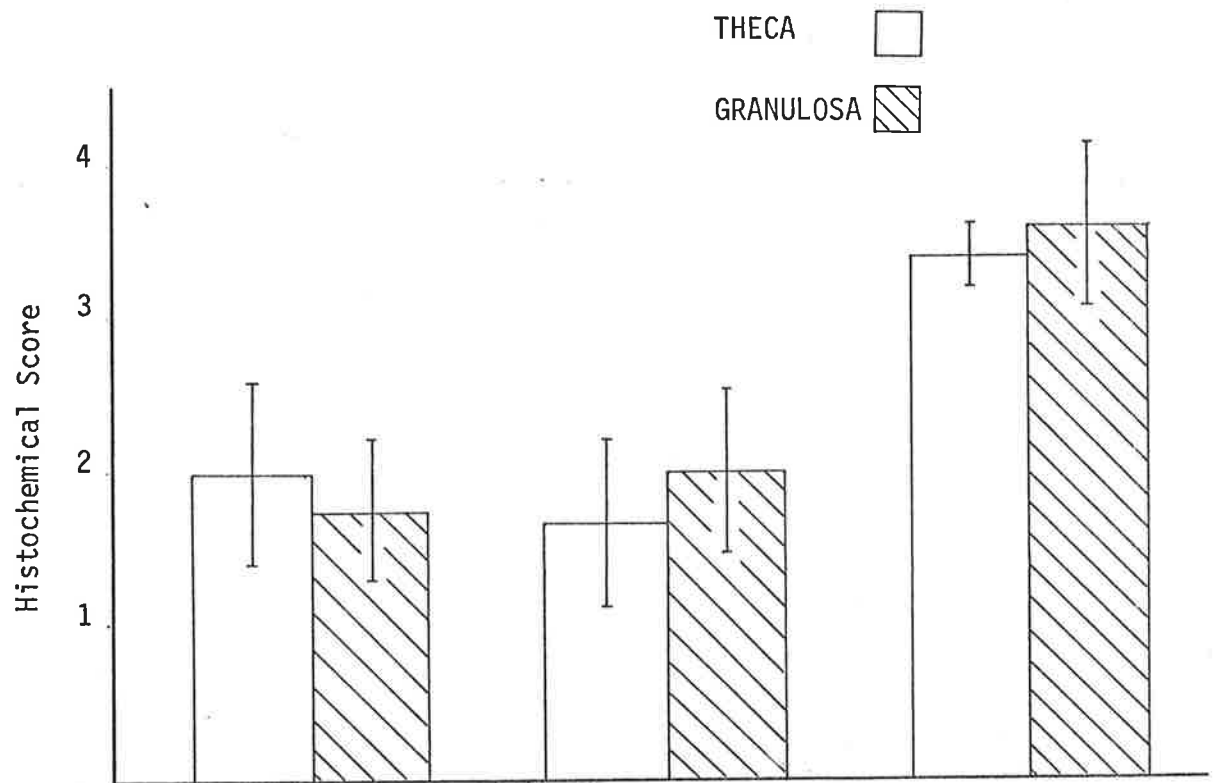
Histochemical localization and activity of 3β -HSD in cultured ovarian follicles (means \pm SEM) following incubation:-

- (1) without the addition of a gonadotrophin (n = 4)
- (2) during incubation with FSH (n = 3)
- (3) during incubation with HCG (n = 5)

These follicles were explanted during the luteal phase (days 15-28) of the menstrual cycle.

The significance of the treatment effects compared with untreated control follicles is indicated by the asterisk * $p < 0.01$.

4.5.3. 3β -HSD activity in luteal phase follicles incubated with gonadotrophins invitro



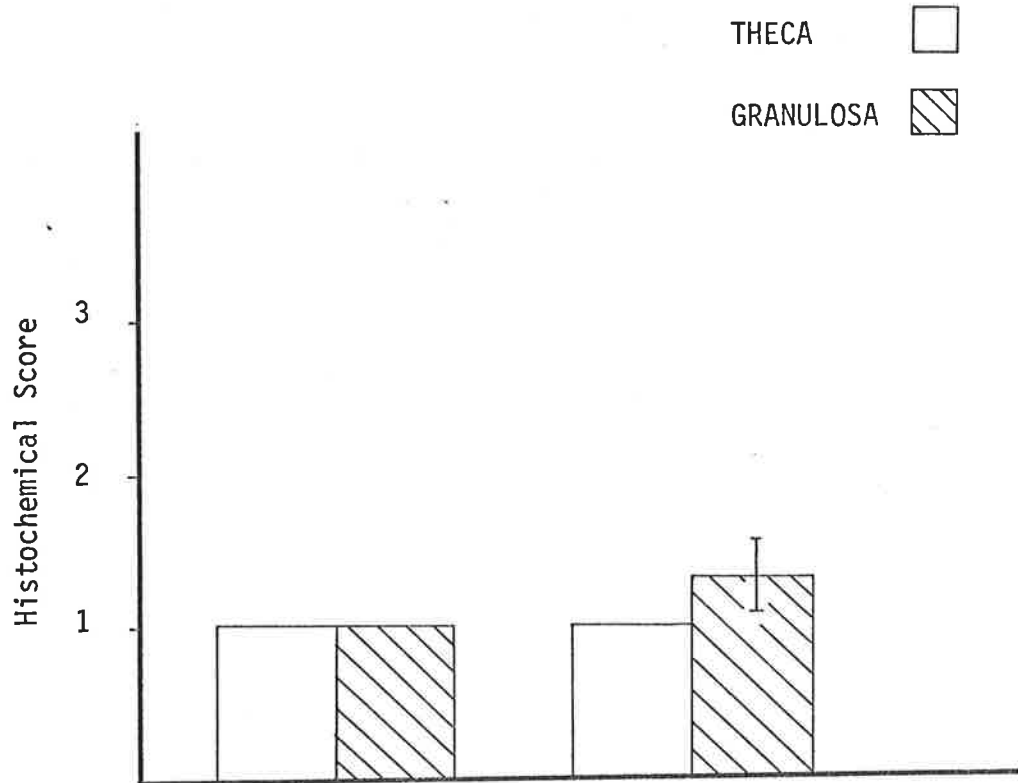
	Control	FSH 20 iu/ml of medium	HCG 20 iu/ml of medium
THECA 3 β -HSD activity (1 \rightarrow 4)	2.0 \pm 0.7 (moderate)	1.7 \pm 0.7 (moderate)	* 3.4 \pm 0.2 (marked)
GRANULOSA 3 β -HSD activity (1 \rightarrow 4)	1.8 \pm 0.5 (moderate)	2.0 \pm 0.6 (moderate)	* 3.6 \pm 0.6 (marked)
Average diameter of follicles (mm)	5.5 \pm 0.5	5.3 \pm 0.2	5.0 \pm 0.1

FIGURE 4.

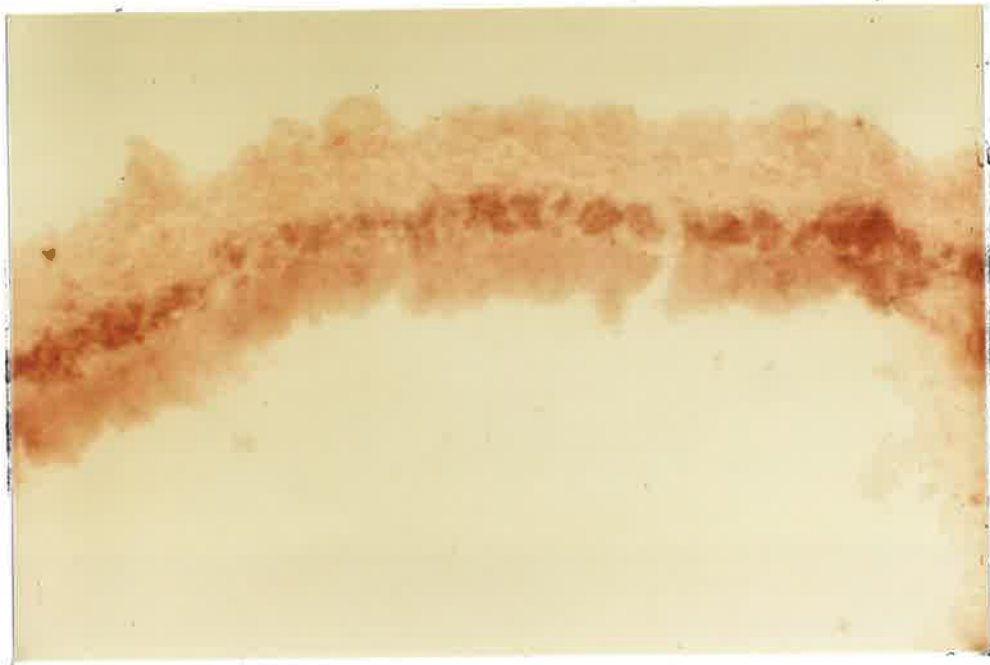
Histochemical localization and activity of 3β -HSD in cultured ovarian follicles (means \pm SEM) following organ culture from follicles explanted during the early post partum period (days 2-5), after incubation with FSH (n = 3) compared to instimulated follicles (n = 4). The steroid output of these follicles during the period in culture preceeding histochemical evaluation is shown in Table 3 of Chapter 3.

4.5.4. Post partum follicles:-

3 β -HSD activity in unstimulated follicles compared to those incubated with FSH



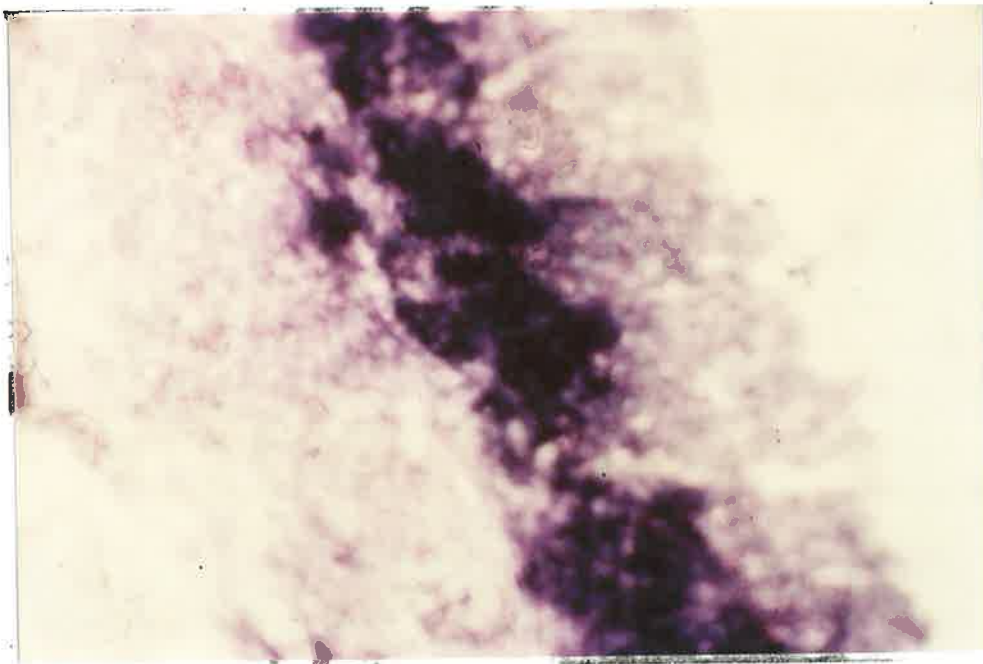
	Unstimulated follicles	Incubated with FSH
THECA 3 β -HSD activity (1 \rightarrow 4)	1.0 (moderate)	1.0 (moderate)
GRANULOSA 3 β -HSD activity (1 \rightarrow 4)	1.0 (moderate)	1.3 \pm 0.3 (moderate)
Average diameter of follicles (mm)	4.2 \pm 0.3	4.1 \pm 0.5



Photomicrograph 15. (x50)

Early follicular phase follicle.

Control - 3β -HSD activity is confined to the theca interna. (Counterstain - 0.2% neutral red chloride).



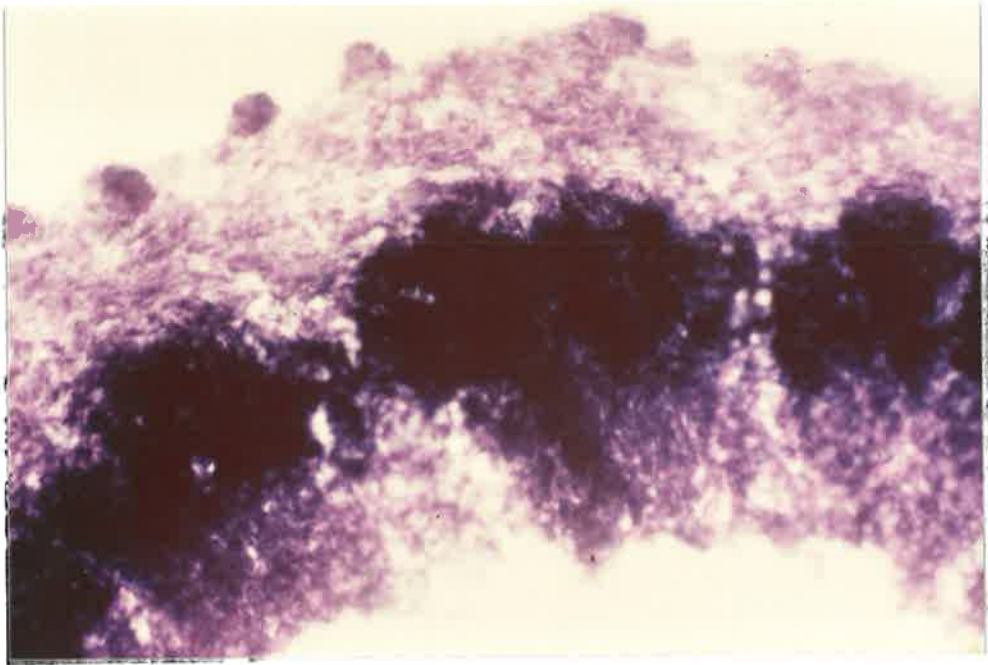
Photomicrograph 16. (x300)

Early follicular phase follicle.

After 24 hours incubation with HCG - 3β -HSD activity is becoming evident in the granulosa layer.

(Counterstain - 2% methyl green).

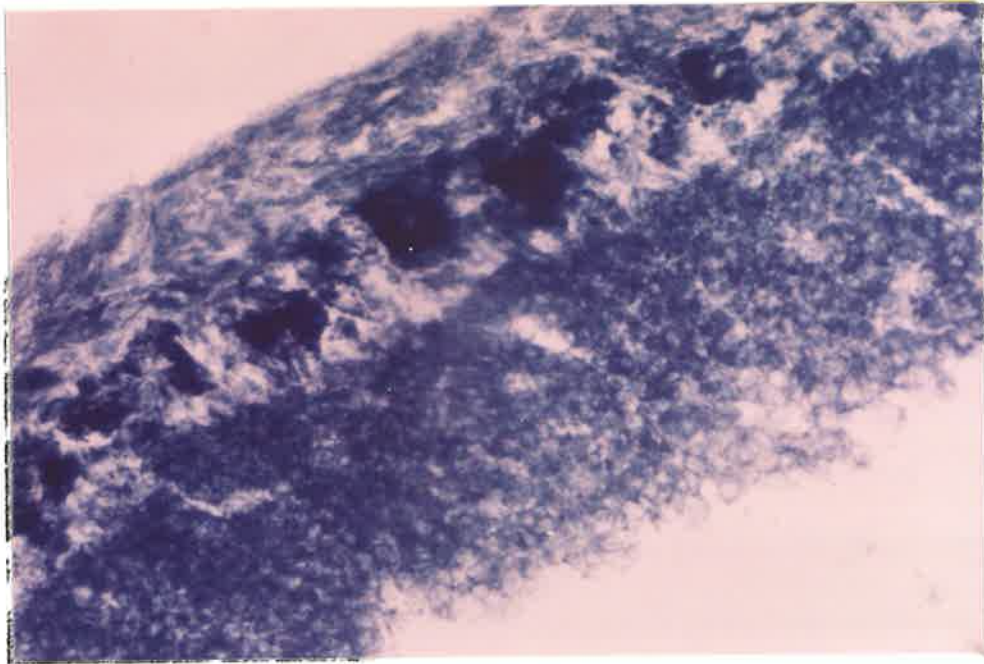
Blue filter in photomicrograph camera.



Photomicrograph 17. (x300)

Early follicular phase follicle.

After 48 hours incubation with HCG - 3β -HSD activity has become more marked in the granulosa cell layer. (2% methyl green and blue filter).



Photomicrograph 18. (x300)

Early follicular phase follicle.

After 72 hours incubation with HCG - 3β -HSD activity has become evident throughout the granulosa cell layer and persistent in the theca interna cell layer. (2% methyl green and blue filter).

4.6. Comparative studies on 3β -HSD activity in follicles incubated with HCG for varying periods of time

Class of follicle

Early follicular phase follicles were used in this experiment because late follicular and luteal phase follicles tend to luteinize spontaneously in organ culture and already have a significant reaction product in their granulosa cells indicating 3β -HSD enzyme activity.

Incubation of early follicular phase follicles with HCG (20 iu/ml)

(Total of 12 follicles were used in this experiment)

3 β -HSD reaction product in	Control follicles (n = 3)	Incubation of follicles with HCG for		
		24 hours (n = 3)	48 hours (n = 3)	72 hours (n = 3)
THECA	2 ++ 2 ++ 2 ++	2 ++ 2 ++ 3 +++	2 ++ 3 +++ 3 +++	3 +++ 3 +++ 2 ++
GRANULOSA	0 - 0 - 1 +	2 ++ 1 + 1 +	3 +++ 2 ++ 2 ++	3 +++ 2 ++ 3 +++
Photomicrograph	15	16	17	18

The results of this experiment indicated that early follicular phase follicles incubated with HCG developed a capacity to acquire significant levels of 3β -HSD activity in their granulosa layers.

This observation correlated with these same follicles ability to produce a significant increased release of progesterone in culture during incubation with HCG (see Table 1 of Chapter 3).

4.7. The pattern of 3 β -HSD activity in late follicular phase follicles following incubation with various substrates

As noted by Wattenberg (1958), DHA and pregnenolone are established and well known substrates for the enzyme, 3 β -HSD. We confirmed this observation with regard to both human and sheep follicles and also noted that 17 α -OH-pregnenolone was also a substrate which could be oxidized by 3 β -HSD. The dynamics of this interaction are illustrated diagrammatically in Diagram 1, 4.2.5.

The substrates were used in the following concentrations:-

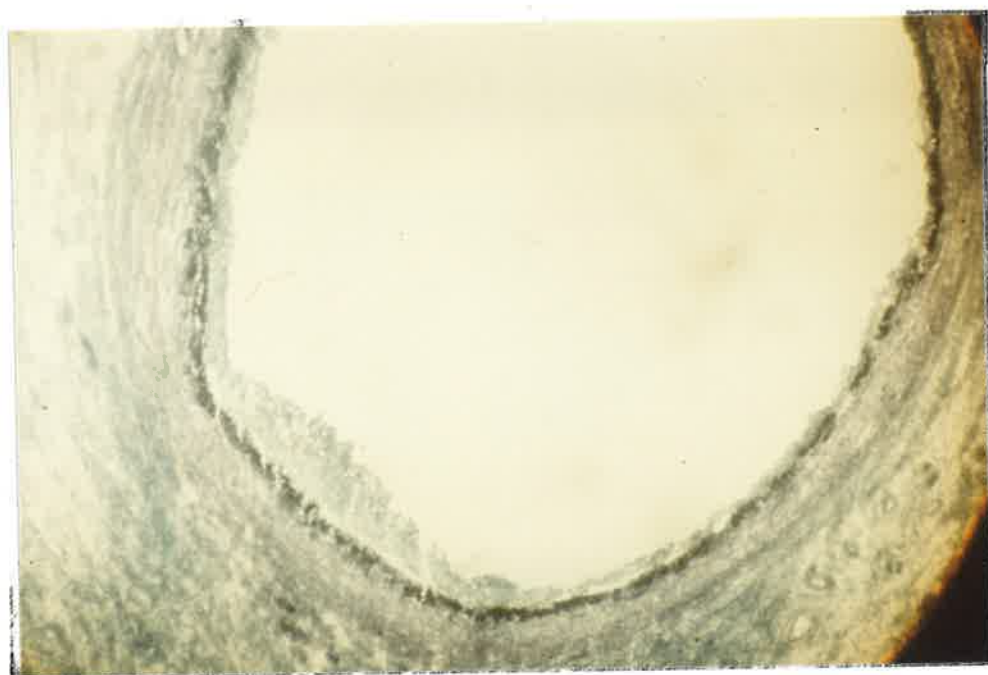
- | | |
|---------------------------------|------------------------------|
| 1. DHA | 6.6 mg/ml of distilled water |
| 2. Pregnenolone | 6.6 " " |
| 3. 17 α -OH-Pregnenolone | 6.6 " " |

An identical section of follicular tissue was incubated in each case without substrate. The other components of the incubation media were identical to those previously described, (see 4.3.1.).

A further section of ovarian stroma was incubated in identical fashion to follicular tissue with each of the substrates.

Follicular phase follicles n = 4	Theca	Granulosa	Stroma
No substrate	0 \rightarrow 1 +	0 \rightarrow 1 +	0 — 0
Pregnenolone	2++ \rightarrow 2++	2++ \rightarrow 2++	0 — 0
17 α -OH-Pregnenolone	2++ \rightarrow 2++	2++ \rightarrow 2++	0 — 0
DHA	2++ \rightarrow 2++	2++ \rightarrow 3+++	0 — 0

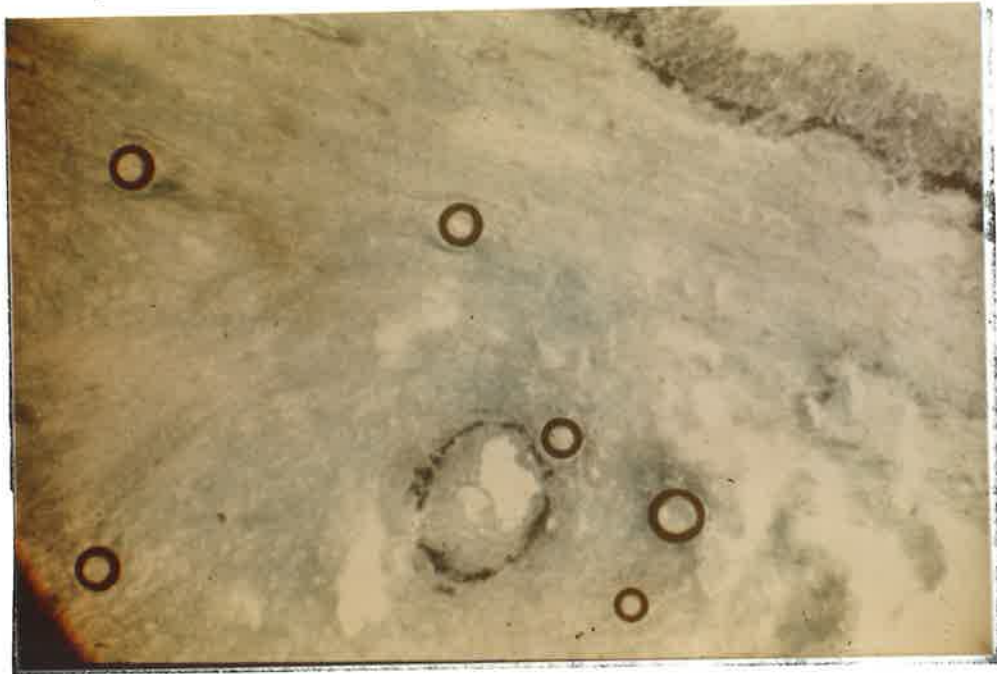
There did not appear to be a preference for a particular substrate tested with regard to 3 β -HSD activity in the theca layer. However there was evidence for selection of DHA as the preferential substrate for 3 β -HSD activity in the granulosa layer of these follicles.



Photomicrograph 19. (x20)

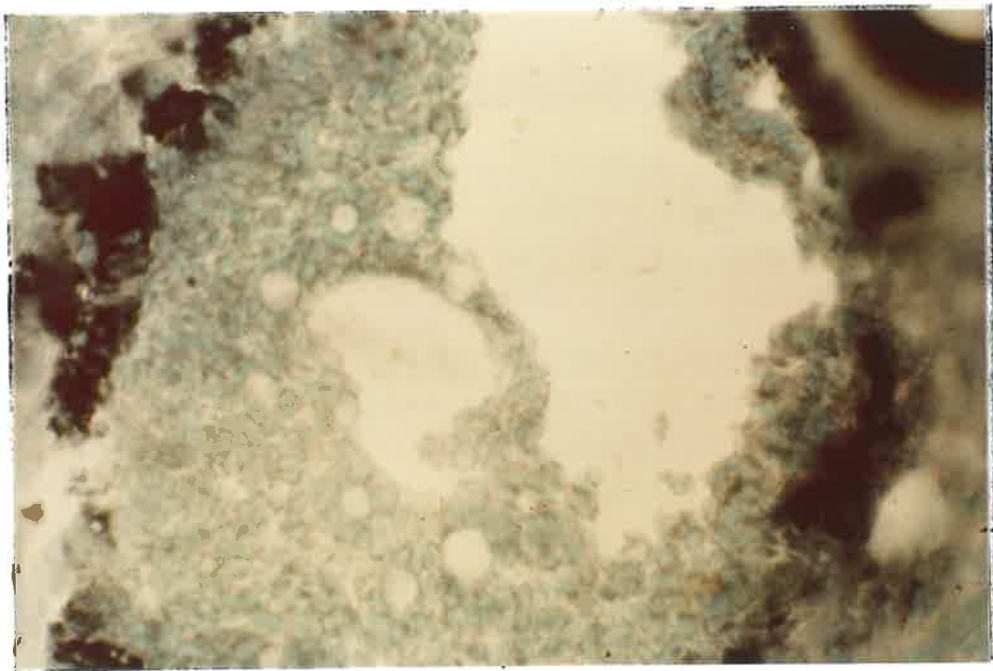
Frozen section of a fresh ovary explanted during the early follicular phase of the menstrual cycle.

3β -HSD activity is confined to the theca interna layer. Refer to 4.8.1. for descriptive details.



Photomicrograph 20. (x20).

Frozen section of a fresh ovary explanted during the late follicular phase of the menstrual cycle. Refer to 4.8.2. for descriptive details.



Photomicrograph 21. (x150)

Histochemical detail of the small antral follicle seen in photomicrograph 20. Refer to 4.8.2. for descriptive details.

4.8. The distribution of 3β -HSD activity in whole ovarian slices, obtained throughout the menstrual cycle

Slices of fresh ovaries obtained at laparotomy were prepared for histochemical evaluation as described for ovarian follicles.

4.8.1. Histochemical localization of 3β -HSD activity in ovarian tissue explanted during the early follicular phase

See photograph 19.

The actual size of this follicle was 5 mm in diameter and the ovary was removed on day 4 of the menstrual cycle. The formazan reaction product is of moderate to marked intensity and clearly confined to the theca interna layer only.

The counterstain was 2% methyl green.

4.8.2. Histochemical localization of 3β -HSD activity in ovarian tissue explanted during the late follicular phase

See photograph 20 and 21.

In the upper right hand corner of photograph 20 is a section of 9 mm diameter follicle. There is a moderate to marked formazan reaction product indicating considerable 3β -HSD activity in both the theca and granulosa layers. In the centre of photograph 20 and enlarged in photograph 21 is a 1.5 mm diameter follicle which has undergone antrum formation. In contrast to its much larger sister follicle it has only a moderate amount of formazan reaction product confined to the theca interna layer. An outline of the ovum and the surrounding cumulus oophorus granulosa cells can be seen. The granulosa cells of follicles less than 3 mm in diameter have been found not to acquire 3β -HSD activity even during the late follicular and luteal phase of the menstrual cycle. It is probable that these follicles are not destined to undergo the final maturational changes in the current cycle but probably will undergo final developmental changes in the next cycle. The 6 dark circular rings are artifacts - air bubbles trapped under the coverslip. The stroma is inactive for 3β -HSD activity.

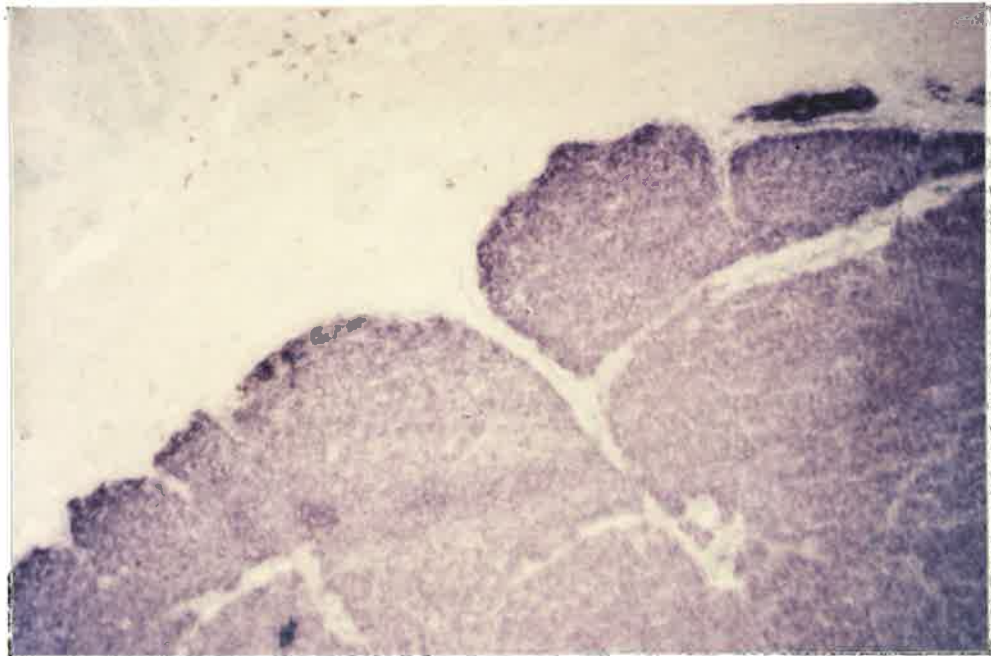
4.8.3. Histochemical localization of 3β -HSD activity in a very early corpus luteum

See photograph 22.

The ovary was explanted on day 14 of a 28 day menstrual cycle. The 'follicle' shown was 13 mm in diameter and has possibly just released the ovum and is undergoing transformation into a corpus luteum.



Photomicrograph 22. (x20)
Histochemical detail of a very early corpus luteum.
Refer to 4.8.3. for descriptive details.



Photomicrograph 23. (x20)
Histochemical detail in a mid luteal phase corpus luteum.
See 4.8.4. for descriptive details.



Photomicrograph 24. (x20)
Total absence of 3β-HSD activity in a corpus albicans.
See 4.8.5. for descriptive details.

Note the early infolding of the follicle wall and the advanced proliferation of the granulosa cells. There is marked formazan deposition in both the theca interna and the entire granulosa layer indicating intense 3β -HSD activity in both theca interna and granulosa cells. The early separation of the granulosa layer from the theca at the level of the basement membrane on the left hand side tends to occur during the histochemical processing.

4.8.4. Histochemical localization of 3β -HSD activity in a mid luteal phase corpus luteum

See photograph 23.

The ovary was removed on day 22 of a regular 28 day cycle. The corpus luteum was bright yellow and had a diameter of 18 mm. A moderate to marked formazan reaction product was uniformly spread throughout the entire section of the corpus luteum indicating that 3β -HSD activity was present in all the steroid active cells of the corpus luteum. Delicate fibrous septae containing blood vessels can also be seen. The surrounding stroma is negative for 3β -HSD activity.

4.8.5. Histochemical studies on a corpus albicans

See photograph 24.

A white outline of a corpus albicans can be seen. Histologically it mainly consists of hyaline and fibrous connective tissue. Of all the intra ovarian components it was the most negative indicator of absent 3β -HSD enzyme activity.

4.9. Correlations between the steroid activity of ovarian follicles invitro and the site of 3β -HSD enzyme activity

The experiments related to the histochemical localization of 3β -HSD enzyme activity in human ovarian follicles were performed immediately after a period of 4 days in organ culture.

For simplicity the steroid profiles of follicles explanted during the early follicular, late follicular, luteal phase and the immediate post partum period and incubated in gonadotrophin free culture media were documented in Chapter 2. Similar groups of follicles incubated with gonadotrophins were documented in Chapter 3. In Chapter 4 the histochemical localization of 3β -HSD activity was described from the follicles examined from a steroidogenic aspect in Chapter 2 and 3.

An attempt has been made to correlate the findings outlined in Chapters 2, 3 and 4 and summarize them in Tables 1 (a), 1 (b), 2 (a) and 2 (b). Although the tables appear complex on first inspection, they are the more simple tables from Chapters 2, 3 and 4 put together to make an integral whole.

Diagram 1 is a simplified outline summarizing the important observations made in relation to follicular size, 3β -HSD enzyme activity, the relative activity of the 3 major sex steroids and the total steroid activity of follicles explanted during the early follicular, late follicular and luteal phase of the menstrual cycle.

Table 1. (a) Steroid release by human ovarian follicles in organ culture and (b) the histochemical localization and activity of 3 β -ol-dehydrogenase. Follicles were explanted during the early follicular phase (days 1-7, n = 5), late follicular phase (days 8-14, n = 6) and the luteal phase (days 15-28, n = 6) of the menstrual cycle, and during the post-partum period days 2-4 after parturition n = 7). See text for details. Asterisks indicate the significance of differences of means compared with late follicular phase follicles where * p<0.1 and ** p<0.05.

a) Steroid secreted into culture medium	Days after explantation	Stage of cycle and steroid output (ng of steroid/mg of follicular tissue per 24 hours)								
		Early follicular		Late follicular		Luteal		Post-partum		
Testosterone	1	23	± 3	19	± 2	21	± 5	76	± 10	**
	2	70	± 21	36	± 10	34	± 7	58	± 9	
	3	41	± 11	22	± 7	22	± 4	28	± 9	
	4	20	± 3	16	± 3	9	± 3	5	± 2	**
Oestrogen	1	14	± 5	30	± 8	10	± 5	45	± 10	
	2	19	± 6	30	± 4	15	± 3	33	± 14	
	3	15	± 5	39	± 7	9	± 2	19	± 5	*
	4	10	± 3	23	± 4	5	± 1	9	± 3	**
Progesterone	1	7	± 2	47	± 21	15	± 3	27	± 7	
	2	9	± 3	119	± 37	18	± 3	35	± 10	*
	3	16	± 9	55	± 21	24	± 2	27	± 6	
	4	6	± 3	48	± 10	18	± 5	13	± 10	**

b) Histochemical localization and activity of 3 β -HSD in ovarian follicles (means \pm SEM) following organ culture. A histochemical score of 0 = no activity, 0-1 = minimal activity, 1-2 = moderate activity, 2-4 = marked activity of the enzyme.

3 β -HSD activity	Early follicular	Late follicular	Luteal	Post-partum
THECA	2.2 \pm 0.3	2.7 \pm 0.4	2.0 \pm 0.7	1.0 \pm 0 **
GRANULOSA	0.7 \pm 0.3 **	2.3 \pm 0.2	1.8 \pm 0.5	1.3 \pm 0.3 **
Average diameter of follicles (mm)	5.4 \pm 0.5 *	8.5 \pm 0.8	5.5 \pm 0.5 *	4.2 \pm 0.3 **

Table 2. Effect of gonadotrophins on (a) steroid release (means \pm SEM) and (b) histochemical localization and activity of 3β -ol-dehydrogenase of human ovarian follicles in culture. Where indicated FSH (20 iu/ml) and HCG (20 iu/ml) were included in media throughout the 3-4 day period in culture. The significance of the treatment effects compared with untreated control follicles (see Table 1) are indicated by the asterisks * $p < 0.01$, ** $p < 0.05$ and *** $p < 0.025$. See Table 1 and text for further details.

a) Steroid released into culture medium	Days after explantation	STEROID RELEASE IN NG/MG OF FOLLICULAR TISSUE PER 24 HOURS									
		Early follicular HCG (n=4)		Late follicular FSH (n=5)		Late follicular HCG (n=4)		Luteal FSH (n=3)		Luteal HCG (n=5)	
Testosterone	1	48 \pm 16 *	25 \pm 6	63 \pm 23 **	14 \pm 8	56 \pm 14 *	72 \pm 9				
	2	97 \pm 39	91 \pm 25	85 \pm 27 *	27 \pm 23	64 \pm 23	72 \pm 6				
	3	48 \pm 18	38 \pm 17	35 \pm 7	35 \pm 15	22 \pm 8	54 \pm 14				
	4	24 \pm 8	28 \pm 16			5 \pm 2	27 \pm 8				
Oestradiol-17 β	1	13 \pm 3	22 \pm 5	7 \pm 4 **	34 \pm 17	24 \pm 10	24 \pm 9				
	2	24 \pm 6	156 \pm 67 *	12 \pm 3 **	34 \pm 15	19 \pm 13	18 \pm 9				
	3	10 \pm 4	46 \pm 17	6 \pm 1 **	11 \pm 5	22 \pm 7	13 \pm 5				
	4	7 \pm 3	31 \pm 11	4 \pm 2 **		8 \pm 4	7 \pm 3				
Progesterone	1	52 \pm 18 ***	19 \pm 5	69 \pm 31	148 \pm 54 **	78 \pm 25 *	103 \pm 41 *				
	2	104 \pm 25 ***	147 \pm 41	92 \pm 26	156 \pm 39 **	53 \pm 11 *	52 \pm 32				
	3	50 \pm 9 **	146 \pm 53 *	49 \pm 3	69 \pm 18 *	91 \pm 28 *	45 \pm 18				
	4	22 \pm 8 *	68 \pm 12	37 \pm 8			29 \pm 8				

b) Histochemical localization and activity of 3β -ol-dehydrogenase in ovarian follicles (means \pm SEM) (n values as above) following organ culture.
 A histochemical score of 0 = no activity, 0-1 = minimal activity, 1-2 = moderate activity, 2-4 = marked activity of the enzyme.

TREATMENT	Early follicular HCG		Late follicular FSH		Late follicular HCG		Luteal FSH		Luteal HCG		Post-partum FSH	
	THECA GRANULOSA	2.5 \pm 0.5	1.6 \pm 0.2	2.3 \pm 0.6	1.7 \pm 0.7	3.4 \pm 0.2*	1.0					
Average diameter of follicles (mm)	2.0 \pm 0.5*	2.2 \pm 0.4	2.3 \pm 0.3	2.0 \pm 0.6	3.6 \pm 0.6*	1.3 \pm 0.3						
	4.6 \pm 0.5	7.1 \pm 1.1	6.1 \pm 0.4	5.3 \pm 0.2	5.0 \pm 0.1	4.1 \pm 0.5						

Diagram 1.

A summary table and diagram outlining the average follicle diameters, relative activity of the enzyme 3β -HSD in the theca and granulosa layers, and comparative testosterone, oestrogen and progesterone release by follicles during the early follicular, late follicular and luteal phase of the menstrual cycle.

The cumulative release of testosterone, oestrogen and progesterone over a 4 day culture period are compared in the early follicular, late follicular and luteal phase. It can be seen that the maximum steroid release per unit mass of follicular tissue occurs in the late follicular phase, followed by the early follicular and then the luteal phase follicles.

Stage of cycle

Early follicular
(days 1-7)

Late follicular
(days 8-14)

Luteal
(days 15-28)

Diameter of follicles (mm)

5.4 ± 0.5

8.5 ± 0.8

5.5 ± 0.5

3β-HSD activity
 { Theca
 Granulosa

Marked

Marked

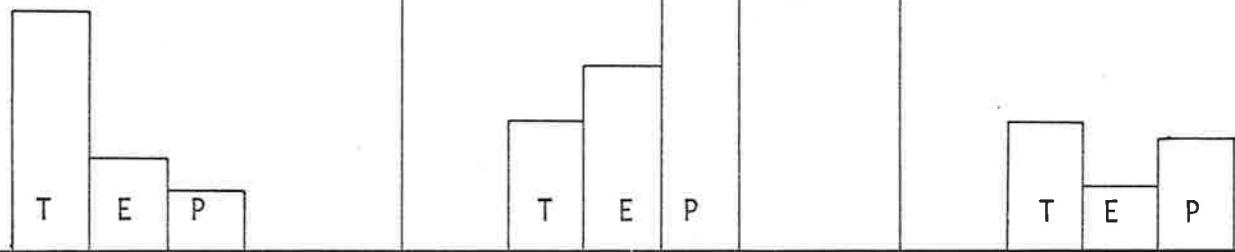
Moderate

Minimal

Marked

Moderate

Steroid release as ng per
mg of follicular tissue
during 4 days in culture



Testosterone (T)
Oestrogen (E)
Progesterone (P)

154

58

38

85

122

269

86

39

75

Total steroid

250 ng

476 ng

200 ng

4.10. Discussion

The histochemical detection of specific enzyme distribution in tissues may be complicated by a number of misleading positive and negative reactions. These procedural problems are partly due to the method employed and to the enzyme one wishes to examine. A number of steps have been taken to overcome many of these problems in order to produce an acceptable degree of credibility to the results and conclusions.

The histochemical method used was one developed by Wattenberg (1958). He was able to demonstrate that the reaction was specific for 3 β -HSD as distinct from other NAD dependent dehydrogenases. Particular care was taken with regard to, (1) the suitability of the tetrazolium salt used, (2) the specificity of the substrate used, (3) the minimal degree of diffusion artifact, (4) the minimal occurrence of any non-specific reaction product was assessed by using a variety of control experiments, (5) lipid extraction prior to incubation was performed as suggested by Wattenberg, (6) the assessment and correlation of histochemical patterns in both fresh and post incubation tissues, (7) observing that 3 β -HSD activity only occurred in tissues where diaphorase activity was also present, and (8) correlating 3 β -HSD activity with the steroid production by the follicular tissue.

This histochemical method has been used by Rahamin, Eshkol and Lunenfeld (1976) in the neonatal rat ovary, by Pupkin, Bratt, Weisz, Lloyd and Balogh (1966) in the adult rat ovary, by Hay and Moor (1975) in the sheep Graafian follicle, by Freedman (1975) in the human ovary during pregnancy and in the post partum period and by Friedrich, Breitenecker, Salzer and Holzner (1974) in the preovular human Graafian follicle. Many of these workers have also correlated 3 β -HSD enzyme activity in the tissue with its steroid activity. However, Novak, Goldberg, Jones and O'Toole (1965), suggested a degree of caution should be exercised in ascribing certain enzyme histochemical activities to known physiologic steroidogenic capabilities of individual tissues or cells. They pointed out that enzymatic activities were not necessarily representative of contained steroids, but only indicated the capacity of the cell for biochemical activity, which under suitable conditions could be involved in hormone synthesis.

In order to overcome this problem an attempt was made to correlate the steroid activity of individual follicles in culture with their dis-

tribution and activity of the enzyme 3 β -HSD. In order to determine if the time in organ culture modified 3 β -HSD enzyme distribution significantly, histochemical studies were also performed on fresh whole ovarian slices to assess 3 β -HSD enzyme activity in follicles at various times throughout the menstrual cycle. There was good histochemical correlation of 3 β -HSD activity in follicles explanted at the same stage of the cycle following four days in organ culture or in fresh ovarian slices.

It has been demonstrated that the smaller early follicular phase follicles had 3 β -HSD enzyme activity almost exclusively confined to their theca interna layer. These same follicles released testosterone predominantly in culture. It was therefore inferred that steroid activity in early follicular phase follicles proceeded by the Δ^5 -pathway in the theca interna cells for the production of androgens. The findings were also consistent with the thesis that aromatase activity for oestrogen production was low and that the Δ^4 - pathway for progesterone production in the granulosa cells was underdeveloped at this stage of the menstrual cycle. However it was shown that incubation of these follicles with HCG induced a significant increased release of testosterone and progesterone and a coincident and significant increased 3 β -HSD activity in their granulosa cells. Histochemical studies on fresh ovarian slices of late follicular and luteal phase ovaries demonstrated that follicles less than 2 mm in diameter failed to luteinize *invivo* whereas their larger counterparts did. In other words these very small follicles had not yet acquired the capacity to respond to HCG.

The larger follicles explanted during the late follicular phase of the menstrual cycle already had histochemical evidence of 3 β -HSD enzyme activity in their granulosa cells and sustained activity of the enzyme in their theca cells. This observation was associated with an increased release of progesterone, oestrogen and androgens in organ culture. Histochemical examination of fresh ovarian slices from ovaries removed during the late follicular and luteal phase also had evidence of moderate to marked 3 β -HSD activity in their granulosa cells and sustained activity in their theca layers. Incubation of late follicular phase follicles with either FSH or HCG did not elicit significant increased 3 β -HSD enzyme activity on histochemical assessment. It was inferred from this observation that the degree of 3 β -HSD activity which was already present in late follicular phase follicles

was sufficient to enable steroidogenesis to proceed via the Δ^4 -pathway. This thesis was supported by the observation that the incubation of these follicles with FSH was associated with a significant increased release of testosterone, oestrogen and progesterone.

Following incubation in gonadotrophin free media, luteal phase follicles had moderate to marked 3β -HSD activity in both the theca and granulosa cells at a time when the steroid activity in these follicles (per unit mass) was decreased. There was evidence of increased 3β -HSD enzyme activity in both the theca and granulosa cell layers following incubation of these follicles with HCG. This observation was associated with a significant increased release of testosterone and progesterone. FSH on the other hand did not alter 3β -HSD activity. The reason for this difference was not apparent. Exposure to HCG may have been associated with an increased formation of 3β -HSD and in turn because of the luteal follicles decreased steroid activity and metabolic rate, accumulation of 3β -HSD may have occurred.

An alternative explanation related to work done by Wiener (1976), on the inhibition and release of 3β -HSD enzyme activity in relation to progesterone production by the mammalian placenta, may be plausible. It was demonstrated that a metabolite of progesterone, 20α -hydroxy-pregn-4-ene-3-one (20-DHP) was associated with noncompetitive inhibition of 3β -HSD activity. However 20-DHP was not found to inhibit progesterone production in homogenates of human corpora lutea. Another substance, as yet unidentified, but found in placental tissue also inhibited progesterone production. To date it has been characterized as being dialysable, lipid soluble and of low molecular weight, which suggested that it too, could be steroid in nature. It was noted that placental damage was related to loss of this inhibitor and increased 3β -HSD activity. This observation could partly explain the increased formazan deposit in luteal follicles subjected to HCG. The combination of increased progesterone release and loss of 3β -HSD inhibitors due to dissection and isolation of the follicle in culture may be contributing factors. However this explanation does not account for the absence of change in 3β -HSD activity when luteal follicles were incubated with FSH under identical conditions.

There was evidence of moderate histochemical activity of 3β -HSD in both the theca and granulosa cells of post partum follicles following organ culture. These follicles were quite active steroidogenically

and comparable to the activity observed in late follicular phase follicles in culture. However from both a histochemical and steroidogenic aspect, they behaved differently following incubation with FSH. Incubation of these follicles with FSH did not produce an alteration in 3β -HSD activity in either the theca or granulosa cells, and was only associated with a transient increased release of progesterone and no alteration in oestrogen or testosterone release. As noted in Chapter 3, these observations on the post partum follicle supported the thesis proposed by Reyes et al., 1977 and Rolland et al., 1975, that the post partum ovary was refractory to gonadotrophin stimulation. Freedman, (1975) suggested that because placental HCG takes a week to be cleared from the maternal circulation following parturition, it may be responsible for ovarian follicular development. He found hyperplasia of theca interna to be common at this time; a feature not noted in this study.

Leavitt, Bosley and Blaha (1971), demonstrated a parallel pre-ovulatory progesterone surge and the development of 3β -HSD activity in the theca and granulosa cells of preovulatory golden hamster follicles. This observation was very similar to the 3β -HSD enzyme-progesterone relationship noted for the human follicle in culture.

Shawky and Badawy (1976) immunized rats against 3β -HSD, which was followed by anoestrus cycles, a decreased histochemical localization of 3β -HSD in corpora lutea and interstitial tissue, failure to conceive and complete resorption of implantation or stillborn fetuses. This work served to illustrate the significance of this enzyme in ovarian steroidogenesis and in particular, the results of immunization, as judged by the nature of the complications, suggested that suppression of progesterone production was a major factor. The histochemical studies in this thesis support these findings. For instance, whenever the follicles released significant levels of progesterone, there was always significant evidence of 3β -HSD activity in the granulosa cells of follicles explanted at any stage of the reproductive cycle. Also destruction of 3β -HSD enzyme activity in sheep corpora lutea by megavoltage irradiation was associated with a marked fall in progesterone secretion by these ovaries. It was of interest to note that Wiener (1971) found an increased activity of the 3β -HSD enzyme in homogenates of human placenta which were subjected to x-irradiation. It was postulated that the x-irradiation may have destroyed an inhibitor of 3β -HSD. The 700 rad dosage used in the experiments reported in this thesis indicated

that an increase in 3β -HSD activity occurred in sheep corpora lutea when compared to control corpora lutea. However it was difficult to judge if the change in 3β -HSD activity was significant as this observation was not associated with an increased progesterone release. In light of Wiener's observations it would have been interesting to subject sheep corpora lutea to smaller doses of irradiation and then assess their steroid and 3β -HSD activity.

In contrast to sheep ovaries, which did not show evidence of 3β -HSD activity in the granulosa cells until a few hours after ovulation (Hay and Moor, 1975), human follicles showed significant 3β -HSD activity in the granulosa cells of late follicular phase follicles. This apparent species difference did correlate with the observation that a rise in 17α -OH-progesterone and progesterone occurred in the human prior to ovulation (Yussman and Taylor, 1970; Friedrich, Breitenecker, Salzer and Holzner, 1974). However, Wheeler, Baird, Land and Scaramuzzi (1975), have shown that a small and transient rise in progesterone secretion did occur in the sheep just prior to ovulation, but to a much lesser extent than in the human. They suggested that this difference may arise from the different patterns of the preovulatory LH surge, that of the sheep lasting for about 12 hours while that of the woman persisted for 2 to 3 days.

The characterization of 3β -HSD activity in the follicle throughout the reproductive cycle and relating it to steroid activity may help to develop a better understanding of ovarian steroid disorders such as polycystic ovarian disease. The mechanism of steroid pathogenesis in polycystic ovarian disease is still unclear. Kase (1964), found that the polycystic ovary produced significantly more androgen than the normal ovary. By using radioactive incorporation studies on incubated whole tissue homogenates from polycystic ovarian wedges, he found that a specific androgen accumulated, which was related to the precursor substrate used during incubation. When progesterone was used as substrate, more androstenedione than testosterone was formed, but when pregnenolone was used more testosterone than androstenedione was formed. It was noted in a previous study (Kerin, Moor and Seamark, 1976), and reported in this thesis, that DHA appeared to be the preferred substrate for 3β -HSD activity in the granulosa layer of human follicles. The implications of substrate specificity could be significant in ovarian disorders where the preferred steroid pathway is impaired. Since DHA secretion by polycystic ovaries was also found to be high and progesterone production low, it may implicate a 3β -HSD enzyme disorder in these follicles

(Kase 1964). It has been shown in this thesis that small early follicular phase follicles produced predominantly androgens and little progesterone in organ culture. Since there are many small follicles present in polycystic ovarian disease, it may be that steroid abnormalities reflect, rather, the excess number of small follicles available for steroidogenesis rather than an actual biosynthetic enzyme 'block'. This conclusion was supported and first proposed by Kase (1964).

4.11. Summary

It has been shown that the histochemical localization of Δ^5 - 3β -HSD enzyme activity in the human ovarian follicle following organ culture, reflected changing steroid activities within the follicle. Considerable care was taken to validate the histochemical procedure. There was a good correlation between the patterns of 3β -HSD activity in fresh ovarian slices and in follicles following organ culture.

In the smaller early follicular phase follicles, 3β -HSD activity was mainly confined to the theca interna cells where the predominant steroid released was testosterone. Late follicular phase follicles had evidence of considerable 3β -HSD activity in both theca and granulosa cells and were the most active follicles steroidogenically, producing large amounts of testosterone, oestrogen and progesterone. Luteal phase follicles had evidence of moderate 3β -HSD activity in both the theca and granulosa cells, but steroidogenically were less active than early follicular phase follicles. Post partum follicles had evidence of moderate 3β -HSD activity both in the theca and granulosa cells and released steroids invitro at a level comparable to late follicular phase follicles.

Incubation of early follicular phase follicles with HCG was associated with the development of 3β -HSD activity in the granulosa cells and an increased progesterone output. Incubation with FSH and HCG did not alter the already established 3β -HSD activity in late follicular phase follicles but in luteal phase follicles HCG enhanced 3β -HSD activity. Incubation of post partum follicles with FSH did not alter 3β -HSD activity, and apart from a transient rise in progesterone output, FSH did not significantly alter these follicles steroid activity.

In this study attention has been drawn to the potential use of this approach to investigate disorders of ovarian steroidogenesis in relation to enzyme-substrate specificity and the ultimate steroid released by ovarian follicles.

5. TESTOSTERONE, OESTROGEN AND PROGESTERONE ACTIVITY IN ISOLATED HUMAN THECA & GRANULOSA CELLS IN TISSUE CULTURE

5.1. Introduction

The ovarian follicle has been implicated as a major source of ovarian oestrogen (Baird, Barker, McNatty & Neal, 1975), but the site of oestrogen production within the follicle has been the subject of much debate. Since Falk (1959) concluded that the combined function of the theca and granulosa cells was necessary for oestrogen production, a great deal of research has been done in an attempt to determine the biochemical basis of this interdependency between the two cell types.

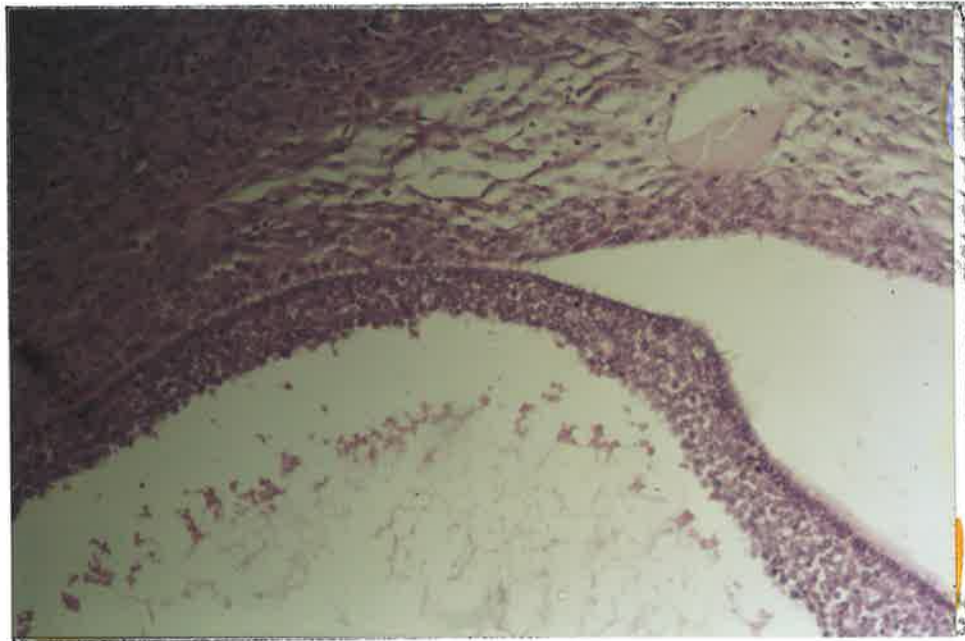
One common approach has been to isolate the theca and granulosa cells and study their steroidogenic capabilities separately in culture. However, these procedures destroy intercellular communications and the cell isolates may not be pure or their function truly representative of the *in vivo* situation. Despite these problems important findings have been made. Pure isolates of human granulosa cells has been achieved (Channing, 1969; Kerin & Seamark 1977), but the production of pure isolates of theca cells has not been as successful. This problem probably accounts for much of the current disagreement about the synthetic capacity of thecal tissue. Channing (1969), was able to show that isolates of human granulosa cells produced predominantly progesterone, and that theca cells produced predominantly androgens and oestrogens, *in vitro*. Armstrong and Dorrington (1977) have proposed that the theca cells produced the androgen substrate which was transported to the granulosa cells for aromatization to oestrogens.

An attempt has been made to determine the intrafollicular sites of steroidogenesis by culturing combined and isolated thecal and granulosal tissue, *in vitro*, from human follicles explanted during the late follicular phase of the menstrual cycle.

5.2. Methodology

Ovarian follicles were removed from the ovaries of 8 women during the operative procedure of abdominal tubal ligation. All of these women were having spontaneous menstrual cycles and were operated on during the late follicular phase of the menstrual cycle (days 8-14) as judged by the date of the last menstrual period and the histological dating of the endometrium obtained by curettage.

The follicles were transported to the laboratory in sterile transport media and underwent immediate microdissection in order to obtain stroma free follicles. The procedure to this stage has been described in detail



Photomicrograph 25.

Spontaneous separation of granulosa layer.

A chance observation showing the line of cleavage between the basal layer of granulosa cells and the innermost theca interna cells at the level of the basement membrane. Intercellular extensions can be seen projecting from the basal layers of granulosa cells. These projections presumably contribute to the intercellular complex of plasma membranes and gap junctions referred to by Moor et al., (1973), page 16.

in Chapter 2.

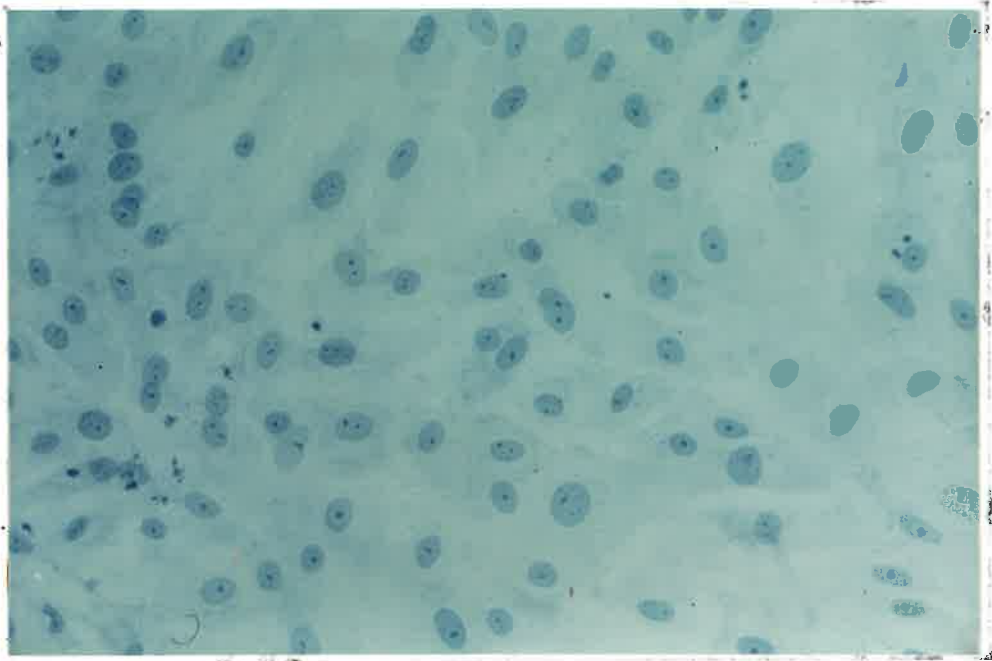
5.2.1. Technique of theca cell and granulosa cell isolation, culture and incubation

- (1) The cleaned ovarian follicle was placed in a Petri dish which contained 3 to 4 ml of dissecting media (see 2.2.5).
- (2) The follicular diameter was recorded and the follicle then divided in half.
- (3) One half of the follicle was left as an intact theca-granulosa cell component and cultured as described for whole follicles (see 2.2.4.).
- (4) The other half was transferred to another Petri dish and separated into its granulosa and theca cell components in the following way:-
- (5) In 1 ml of dissecting medium the half follicle was placed on the bottom of the dish with its granulosa cells facing uppermost.
- (6) One edge of the tissue was held with a fine pair of watchmaker forceps and the granulosa cells gently scraped off the 'theca shell' layer with a sterile platinum wire loop. There appeared to be a natural plane of separation at the junction of the theca and granulosa cells at the level of the basement membrane. See photomicrograph 25.
- (7) The remaining granulosa cells were removed by directing a strong current of dissecting media over the tissue, using a 23 gauge needle attached to a 5 ml syringe.
- (8) After adding a few drops of dissecting medium onto the 'theca shell' it was examined under a dissecting microscope to see if any clumps of granulosa cells were still attached.
(It has been suggested by Armstrong (1977), that if there was still evidence of persistent adherence of granulosa cells to the 'theca shell', then the tissue should be placed in a solution of trypsin (0.25% in PBS) or testicular hyaromidese, No. 361272 (diluted to a concentration of 10 iu/ml of PBS), to free the remaining granulosa cells. This step was not used in the procedure described. However follow up histology of the 'theca shell' showed only minimal evidence of small areas of remaining granulosa cells.
- (9) The 'theca shell' was removed, rinsed, and then cultured as described for whole follicles (see 2.2.4.).

- (10) The freed granulosa cells suspended in about 2 ml of dissecting media were poured into a sterile tube containing 20 mg of dried ethylene diamine tetra acetate (EDTA), to prevent them from clumping together.
 - (11) The suspended granulosa cells were then centrifuged at 600 r.p.m. for 5 minutes.
 - (12) The supernatant was decanted off and the granulosa cells re-suspended in dissecting media to remove any remaining EDTA.
 - (13) The suspension of granulosa cells was again centrifuged at 600 r.p.m. for 5 minutes, and the supernatant poured off.
 - (14) The cells were resuspended in 0.25 ml of culture medium.
 - (15) They were then seeded onto a sterile coverslip in a large Petri culture dish.
 - (16) They were left at 37°C for 1½ to 2 hours to enable the cells to adhere to the coverslip. The degree of adherence could be assessed with the inverting microscope.
 - (17) When granulosa cell adherence was judged to be adequate, the Petri dish was gently flooded with 1.75 ml of culture medium.
 - (18) The Petri dish cover was added to the granulosa cell monolayer which was then cultured in exactly the same way as described for whole follicles (see 2.2.4.).
 - (19) The culture media was aspirated at 24 hour intervals and replaced with fresh media for a period of 3 days.
 - (20) The culture media content of testosterone, oestradiol-17β and progesterone was estimated by using the validated radioimmunoassay procedures as described in 2.2.8.
 - (21) The intact theca-granulosa cell components, the 'theca shells' and the granulosa cell monolayers were all harvested at the end of day 3 in culture and processed for histological assessment.
- 5.2.2. Assessment of the weight of granulosa cell monolayers, theca tissue isolates and intact theca-granulosa tissues

- (1) Calculation of the weight of the intact theca-granulosa tissue component

Each follicle's diameter was recorded. Since the follicles were carefully cut into halves their wet weight was calculated from the tables of the regression of wet weight on diameter (see 2.2.11.), and divided by 2.



Photomicrograph 26.

Granulosa cell monolayer after 3 days in tissue culture. The cells are morphologically viable and there is no evidence of contamination with theca cells or other cells.

(2) Calculation of the 'theca shell' weight

This was calculated by subtracting the estimated granulosa cell weight from that of the half follicle weight, having determined its diameter. The ratio of theca to granulosa layer thickness was calculated for a series of follicles ranging from 3 to 12 mm in diameter (see 2.2.12.).

(3) Calculation of the weight of the granulosa monolayers

The average diameter of a granulosa cell was calculated and found to be $16.4 \mu \pm 0.2 \mu$ (SEM), see 2.2.12.

The granulosa cells were spread evenly over the coverslip as a uniform single layer of cells. There was no contamination from theca cells or other foreign tissue. See photomicrograph 26.

The granulosa cells were therefore easy to count with the help of a counting chamber grid. Ten areas of granulosa cells on each coverslip were counted, the average number per unit area determined so that the total number of cells on each coverslip could be determined. The number of cells on each coverslip ranged from 30,000 to 65,000 and represented the number of granulosa cells cultured for each patient.

Assuming that the granulosa cell density was very close to 1, one could say that their cell mass was equal to their volume.

$$\begin{aligned} \text{Therefore the volume of a granulosa cell} &= \frac{4}{3} \pi r^3 \\ &= \frac{4}{3} \pi (8.2)^3 \\ &= 2309 \text{ cu} \end{aligned}$$

The weight of the granulosa cell monolayer =
($2309/10^9$ x number of cell on the coverslip) in milligrams.

The amount of steroid released from the intact theca-granulosa, theca shell only and the granulosa monolayer was expressed as ng of steroid per mg of follicular cell tissue per 24 hours.

5.3. Results

The average age of the women from whom follicles were obtained was 34 ± 2.4 years (SEM, $n = 8$).

The average diameter of the follicles was $7.4 \text{ mm} \pm 0.6$ (SEM, $n = 8$).

5.3.1. Comparative steroid activity of intact theca-granulosa follicular tissue, theca tissue and granulosa cell monolayers, invitro. See Table 1 and Figure 1

- (1) Testosterone release by intact theca-granulosa tissue and isolated theca tissue was high. The release of testosterone from the intact theca-granulosa and isolated theca tissue, on a weight for weight basis was comparable. However, testosterone release by the granulosa monolayers was significantly lower than that released by either the intact theca-granulosa or theca only tissue ($p < 0.025$ on days 1, 2 and 3 in tissue culture).
- (2) Oestrogen release by the intact theca-granulosa tissue component was higher than either the theca or granulosa components in isolation. Oestrogen release by the intact theca-granulosa component was significantly greater than the isolated theca component ($p < 0.1$ on day 2 in culture), and also significantly greater than the isolated granulosa cell component ($p < 0.05$ on days 2 and 3 in culture). The oestrogen released by the addition of the isolated theca and granulosa components did not reach the total oestrogen released by the intact theca-granulosa component.
- (3) Progesterone release by the intact theca-granulosa tissue component steadily rose from day 1 to 3 in tissue culture. The increased progesterone output on day 3 was significantly different from the output on day 1 in culture ($p < 0.025$). Progesterone release by the isolates of theca tissue was significantly less than that of the intact theca-granulosa tissue ($p < 0.025$ on day 3 in culture) and also significantly less than that released by granulosa cell monolayers ($p < 0.1$ on days 1, 2 and 3 in culture). Progesterone release by the intact theca-granulosa component was comparable to that of the granulosa monolayer component. In both tissue components progesterone release increased in relation to the time in culture for up to 3 days.

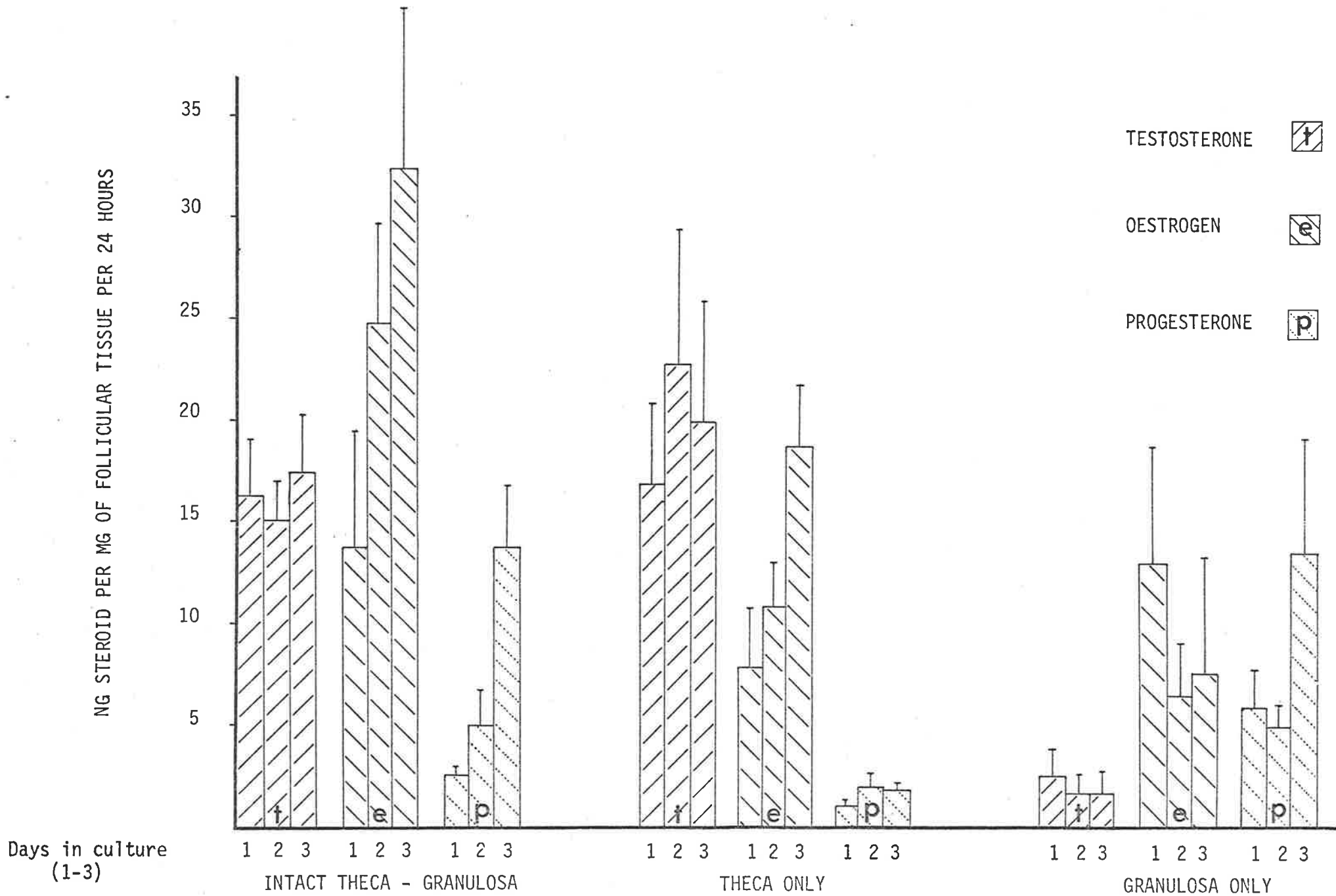
Table 1. Release of testosterone, oestrogen and progesterone (means \pm SEM) by the intact theca-granulosa follicle wall (n = 8), theca shell (n = 4) and granulosa cell (n = 4) maintained in culture. Tissues were obtained from follicles explanted during the late follicular phase of the menstrual cycle.

Steroid secreted into culture medium	Days after explantation	STEROID OUTPUT IN NG/MG FOLLICULAR TISSUE PER 24 HOURS								
		Theca-Granulosa			Theca			Granulosa		
Testosterone	1	16	\pm	3	17	\pm	4	3	\pm	1.5
	2	15	\pm	2	23	\pm	7	2	\pm	1
	3	17	\pm	3	20	\pm	6	2	\pm	1.2
Oestrogen	1	14	\pm	6	8	\pm	3	13	\pm	6
	2	25	\pm	5	11	\pm	2	7	\pm	2.6
	3	32	\pm	9	19	\pm	3	8	\pm	6
Progesterone	1	2	\pm	0.4	0.9	\pm	0.4	6	\pm	1.8
	2	4	\pm	1.8	1.7	\pm	0.6	5	\pm	1
	3	13	\pm	3	1.6	\pm	0.3	14	\pm	6

FIGURE 1.

Steroid released expressed as ng of steroid per mg of (1) intact theca-granulosa tissue, (2) isolates of theca tissue and (3) granulosa cell monolayers in tissue culture per 24 hours, for a total of 3 days incubation.

The testosterone, oestrogen and progesterone profiles of the individual follicular tissues in tissue culture can be seen. The SEM is shown for each steroid released per 24 hours in tissue culture.



5.4. Discussion

It has been demonstrated that the separated cellular components of the human Graafian follicle could be maintained in tissue culture with the maintenance of steroid activity characteristic of the isolated cell types being studied.

Follicles were explanted from the ovaries of women during the late follicular phase of the menstrual cycle (days 8-14) for this study because it was noted from previous observations (see Chapter 2), that these follicles were the most active steroidogenically in culture, and also likely to represent the steroid activity most characteristic of the preovular follicle. In a previous study by McNatty, Hunter, McNeilly and Sawers (1975), it was shown that the granulosa cells harvested from late follicular phase follicles (i.e. 'active follicles') secreted maximum amounts of progesterone within 2-6 days in culture, probably because they had been exposed to high levels of FSH and oestradiol-17 β in follicular fluid.

Testosterone released by isolated theca tissue was similar to that produced by intact theca-granulosa tissue. Testosterone released by the isolates of granulosa cells was very low. These findings were consistent with the thesis that androgens were almost exclusively produced by the theca interna cells. Similar observations have been reported by Channing (1969) in the human follicle and by Fortune and Armstrong (1977) in the proestrus rat follicle.

Oestradiol-17 β released by the intact theca-granulosa tissue was high and lesser but almost equivalent amounts were released by isolates of theca and granulosa cells. This observation supported Falck's (1959) original observation that the two types of follicle cells were necessary for optimal oestrogen production in microtransplants of the rat ovary. Ryan and Petro (1966) were able to show that both the theca and granulosa cells were able to convert pregnenolone and progesterone to estrone and estradiol-17 β in the human follicle. Furthermore, Ryan, Petro and Kaiser (1968), were able to show that the recombination of separated granulosa and theca cells augmented the incorporation of radioactive acetate to oestrogens. Suzuki, Mori and Fujita (1976), were able to show that oestrogen synthesis increased in proportion to proliferation of granulosa cells in the human follicle. This marked proliferation of granulosa cells in the human preovular follicle and early corpus luteum

has been noted by many workers and can be clearly seen in photomicrograph 22 which displays the transition of a post ovular follicle into an early corpus luteum. Armstrong and Dorrington (1977) have produced evidence to support the concept that the theca interna cells of the rat follicle produced androgens which were transported to the granulosa cells for aromatization to oestrogen. McNatty, Baird, Bolton, Chambers, Corker and McLean (1976) have noted that the large preovular human follicles which contained high levels of FSH, also contained high levels of oestrogen and low levels of androstenedione. This observation also supported the concept that granulosa cells of late follicular phase follicles had the ability to aromatize androgens to oestrogens.

The observations in this thesis have indicated that a parallel rise of oestrogen and progesterone release occurred in cultured human follicles in relation to their increased size, the appearance of 3β -HSD activity in their granulosa cells and their nearness to approaching ovulation at the time of explantation. It has been shown that LH receptor activity of granulosa cells increased in relation to follicular size whereas their FSH receptor activity decreased, (Nakano, Akahori, Katayama and Tojo, 1977). This observation provided further indirect evidence that the preovular granulosa cells had acquired the receptor activity necessary to respond to the impending surge of LH. These studies on intact and isolates of theca and granulosa cells in tissue culture supported these observations and provided evidence which indicated that the theca cells were primarily responsible for androgen production and that intact theca and granulosa tissue for optimal oestrogen production.

Studies on isolates of the human granulosa cells indicated that they were the primary source of progesterone synthesis and release. The isolates of theca cells produced very little progesterone and the intact theca-granulosa tissue did not produce a greater amount of progesterone when compared to the isolates of granulosa cells. This observation indicated that there was no interdependence for progesterone production between the theca and granulosa cells in late follicular phase follicles. This finding was in contrast to oestrogen production in which most of the evidence pointed to a cooperativity between the theca and granulosa cells for its optimal production.

The histochemical and steroid studies done on late follicular phase intact follicles, invitro, indicated that their granulosa cells had

acquired 3β -HSD enzyme activity and the capacity to produce progesterone. Human follicles with high follicular fluid levels of oestradiol and critical levels of LH, FSH and prolactin had the capacity to produce high levels of progesterone (McNatty and Sawers, 1975). These observations indicated that these were also late follicular and probably preovular follicles which had activated granulosa cells. Steroid activity by isolates of granulosa cells obtained from the ovaries of hypophysectomized immature rats depended upon the inclusion of testosterone and FSH in the incubation media. It was concluded, therefore, that FSH regulated oestrogen biosynthesis in these granulosa cells by the stimulation of the aromatizing enzyme system (Dorrington, Moon and Armstrong, 1975). In contrast to these follicles from immature rats, the cell isolates from late follicular phase follicles presented in this study were able to produce significant levels of steroids spontaneously in culture. These observations indicated that maturational changes had occurred to enable these tissue isolates from late follicular phase to produce characteristic steroid profiles, invitro.

It was likely that these late follicular phase follicle granulosa cells had been exposed to sufficiently high levels of FSH, LH and oestradiol, invivo, for long enough to induce LH receptor activity (Nimrod, Tsafiri and Lindner, 1977), and progesterone synthesis (Goldenberg, Bridson and Kohler, 1972) for further spontaneous steroid activity in tissue culture. Hillier, Knazek and Ross (1977) have proposed that the exposure of immature rat granulosa cells to androgens was necessary if they were to acquire the ability for progesterone production. It has been shown that androgen production by the theca cells of early follicular phase follicles (see Chapter 2, 2.3.2.) was high and may have had a direct effect on the neighbouring granulosa cells to effect subsequent progesterone production in late follicular phase follicles.

5.5. Summary

In vitro, the steroid release by isolates of theca and granulosa cells obtained from late follicular phase follicles, indicated that the theca was the primary source of testosterone synthesis and the granulosa cells the primary source of progesterone synthesis. Isolates of both theca and granulosa cells were able to produce oestrogen, indicating that both cell types may contribute to oestrogen synthesis within the follicle, thereby lending support to the two cell theory for optimal oestrogen production. Evidence has been provided to indicate that the late follicular granulosa cells have acquired 3β -HSD enzyme activity, from studies of whole follicles in culture and the ability to produce significant quantities of progesterone following their isolation in tissue culture.

CHAPTERS 6, 7, 8, 9 & 10 are included to indicate areas of interest that were still being investigated at the time of submission of this thesis.

6. THE ASSESSMENT OF STEROID ACTIVITY AND THE LOCALIZATION OF 3β - Δ^5 -HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN SLICES OF POST MENOPAUSAL OVARY, INVITRO

6.1. Introduction

Evidence for steroid production by the human menopausal ovary has come to the fore only in recent years. It would be useful to know if the postmenopausal ovary fulfills a useful endocrine function or not, because from a clinical point of view this organ is often removed 'routinely', even if macroscopically normal, in peri and post menopausal women if a hysterectomy is being performed. The reason for oophorectomy at this time being to remove a functionally redundant organ which still has the propensity for pathological change.

The actual sources and their relative contributions to oestrogen, androgen and progesterone production in the postmenopausal woman is still unclear. Much investigation has been done to investigate the source of postmenopausal oestrogen production in the ovary (Baird and Guevara, 1969; Judd, Judd and Lucas, 1974), in the ovary and adrenal (Greenblatt, Colle and Mahesh, 1976) and at more peripheral sites (Grodin, Suteri and MacDonald 1973).

A small study has been done which involved the measurement of steroids produced by small slices of postmenopausal ovary in tissue culture and localizing 3β -HSD enzyme activity in tissue from the same ovaries.

6.2. Materials & Methods

Postmenopausal ovaries were obtained from 3 women during the procedure of abdominal hysterectomy and bilateral salpingoophorectomy.

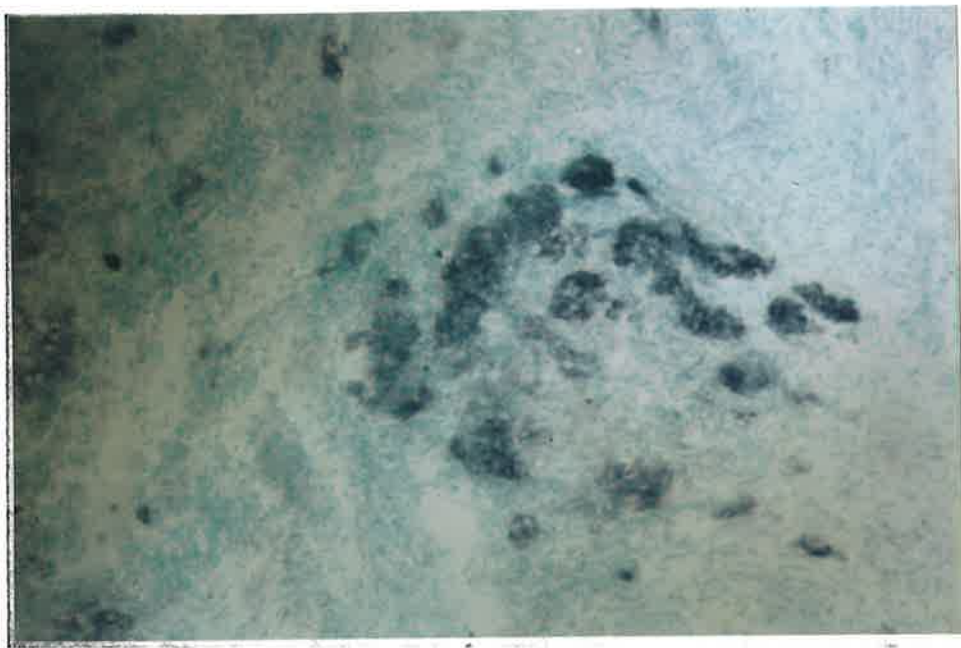
The fresh ovaries were transported to the laboratory under sterile conditions. Small thin ovarian slices (1 x 3 x 0.5 mm) were taken from the ovarian cortex, placed on stainless steel grids and incubated in culture media under identical conditions as those described for whole ovarian follicles (Chapter 2, 2.2.4.). The culture media was aspirated from the tissue slices every 24 hours over a period of 3 days and assayed for testosterone, oestradiol- 17β and progesterone (2.2.8.).

A similar slice of each ovary was frozen and processed to determine any sites of 3β -HSD activity by the methods already described (Chapter 4, 4.3.2.).

6.3. Results

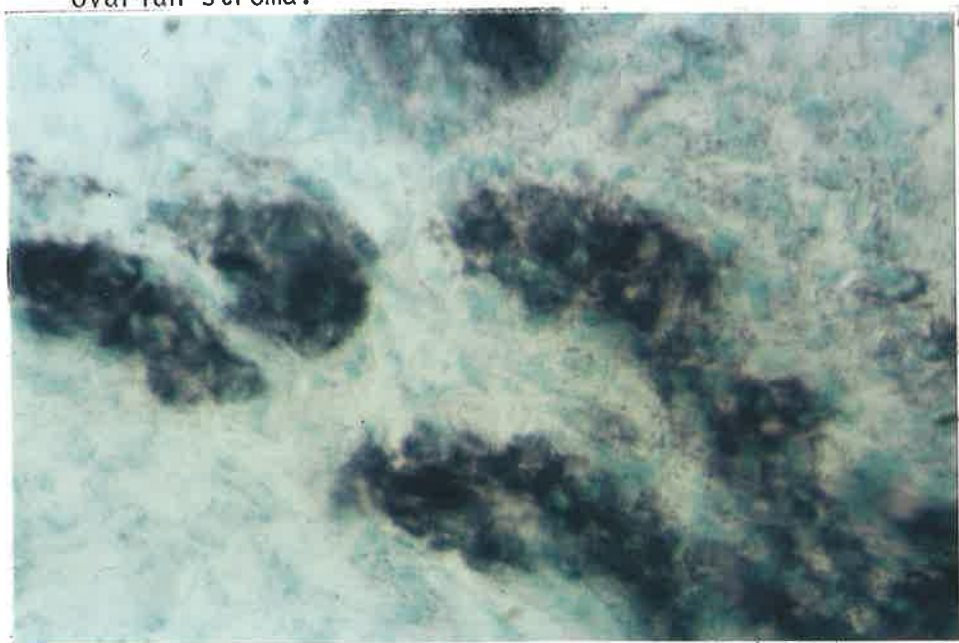
6.3.1. Steroid activity from slices of postmenopausal ovary, invitro

Patient	Clinical details				Steroid activity		
	Age	Months since menopause	Description of ovaries	Pathology	Steroid release expressed as ng of steroid per mg of follicular tissue per 24 hours		
					Testosterone	Oestradiol-17 β	Progesterone
1	52	18	small atrophic	postmenopausal bleeding, large degenerative submucus fibroid	0.5	0	0.7
2	50	15	small atrophic	large fibroid uterus	4.0	0	1.2
3	49	13	small atrophic	postmenopausal bleeding, fibroid uterus	1.0	0	0.3



Photomicrograph 27 (x200)

Circumscribed areas of 3β -HSD activity in postmenopausal ovarian stroma.



Photomicrograph 28 (x600)

Enlarged view of photomicrograph 27. Unfortunately cellular detail cannot be seen using these histochemical methods. However the distinction between inactive and active tissues for this enzyme are almost absolute.

6.3.2. Histochemical assessment of 3 β -HSD activity in postmenopausal ovarian slices

See photomicrographs 27 & 28

Distinct small areas of moderate to strong formazan deposition are seen between large areas of formazan negative ovarian tissue. These formazan positive areas are indicative of localized areas of 3 β -HSD activity.

Histological examination of the stromal slices following culture showed maintenance of cellular integrity, which was consistent with the typical histological features of postmenopausal ovaries.

6.4. Discussion

Although the study was of a preliminary nature it does support the findings of previous workers who have also produced evidence to show that the postmenopausal ovary was steroidogenically active. Because these ovaries were devoid of any structures resembling ovarian follicles or corpora lutea, one may assume that the steroid activity was a product of the ovarian stroma. The corpora albicans present were in advanced stages of regression and as has been previously shown (Chapter 4, 4.8.5.), were the least active intraovarian component for 3 β -HSD activity in the pre and postmenopausal ovary. Therefore they could be ruled out as the origin of steroid activity in the postmenopausal ovary.

Of the steroids measured, testosterone, and to a lesser extent, progesterone, were the only steroids released by postmenopausal ovarian stroma. Further evidence for steroid activity in these ovaries was advanced by finding defined areas of 3 β -HSD enzyme activity, scattered throughout the ovarian cortex.

Grodin, Süteri and MacDonald (1973), have produced convincing evidence which indicated that the major steroid produced by the postmenopausal ovary was Δ^4 -Androstenedione. The steroid pathway to androstenedione could have either been by way of the Δ^5 -pathway via dehydroepiandrosterone or the Δ^4 -pathway via progesterone. Both of these pathways involve the NAD dependent enzyme, 3 β -HSD for the production of androstenedione and ultimately for testosterone and oestradiol production (see Chapter 4, diagram 1). Therefore there was good steroidogenic evidence to support the finding of 3 β -HSD activity in the postmenopausal ovary. Greenblatt, Colle and Mahesh (1976) have shown that the administration of intravenous HCG to post-

menopausal women increased ovarian steroid activity from a comparison of ovarian and peripheral venous steroid levels. Judd, Judd and Lucas (1974) concluded that the postmenopausal ovary continued to secrete a large amount of testosterone, a moderate amount of Δ^4 -androstenedione and low levels of oestradiol-17 β (unfortunately the assay was not available at the time to measure Δ^4 -androstenedione).

The interesting finding was that the postmenopausal ovarian slices invitro, produced significant levels of testosterone but no detectable oestradiol-17 β . It was concluded that the human ovarian stroma was inactive for the aromatization of androgens to oestrogens. This observation supported the evidence produced by Mattingly and Huang (1969), that the endocrine effect of the postmenopausal ovary may be related to its contribution of androgens to the plasma pool for extragonadal conversion to oestrogens.

Gonadotrophin binding sites for both FSH and HCG have been demonstrated in both the cortical stroma and hilus cells of the human postmenopausal ovary (Peluso, Steger, Jaszczak and Hafey, 1976). This observation provided further evidence to indicate that the postmenopausal ovary was steroidogenically active and had the potential capacity to respond to gonadotrophins. In 1964, Scully and Cohen concluded that there was sufficient experimental evidence available to say that 'enzymatically active stromal cells', were present in the postmenopausal ovary. The exact origin of these steroid active cells is still in doubt. It was thought that these islands of polyhedral acidophilic cells may be of adrenal rest cell origin, theca cell remnants of previous follicles or from ovarian hilus cells (Novak, Goldberg, Jones and O'Toole, 1975).

6.5. Summary

The preliminary observations reported, indicated that the human postmenopausal ovary was steroidogenically active. The major steroid isolated from postmenopausal ovarian slices, invitro, was testosterone. A small amount of progesterone was also released in culture. Since oestradiol-17 β was not released by these ovarian slices invitro, this observation supported the thesis that these ovaries lacked aromatase activity and that androgens were the main steroids released from the postmenopausal ovary. These findings were consistent with the concept that peripheral conversion of androgens to oestrogens occurred (possibly in fatty subcutaneous tissue). Circumscribed areas of cells in the ovarian cortex were histochemically active for 3 β -HSD activity, which indicated that they were probably the source of testosterone and progesterone synthesis.

7. RELAXIN ACTIVITY WITHIN THE HUMAN GRAAFIAN FOLLICLE

7.1. Introduction

Relaxin is a polypeptide hormone which is produced by the ovaries and/or reproductive tracts of many animal species during pregnancy. In rats relaxin is secreted by the ovaries during the last week of pregnancy and its most obvious effect was to soften and dilate the uterine cervix, elongate the interpubic ligament and possibly contribute to the onset of parturition by antagonizing the 'progesterone block' to coordinated uterine contractions (Steinetz, Butler, Sawyer and O'Byrne, 1976). Relaxin has also been identified in the blood of female pigs, guinea pigs, rabbits and human beings, increasing from low levels in early pregnancy to high levels in mid to late pregnancy (Sherwood, Chang, Bevier and Dziuk, 1975).

Based on histological and bioassay studies with the pregnant pig, Belt, Anderson, Cavazos and Melampy (1971), suggested that relaxin was stored in cytoplasmic granules within the granulosa cells of corpora lutea and released into the blood stream throughout most of the pregnancy but in increasing amounts for a few days preceding parturition.

Although it has been known that relaxin is a polypeptide hormone for nearly 50 years, an understanding of its site of origin, secretion rates and mechanism of action has been incompletely investigated because the guinea pig and mouse pubic symphysis bioassays have lacked both precision and sensitivity (Sherwood et al., 1975).

A rapid step forward has been made towards understanding the physiological roles of relaxin, since the development of specific and sensitive radioimmunoassays for the estimation of relaxin (Bryant, 1972; Sherwood, Chang, Bevier and Dziuk, 1975; O'Byrne and Steinetz, 1976).

As a result of Braynt, Panter and Stelmasiak's work (1975) on the variation in immunoreactive relaxin in human serum during the menstrual cycle it was considered worthwhile to see if human ovarian follicles in organ culture released relaxin and if so, if their rate of release varied significantly in relation to the time of explantation during the menstrual cycle.

7.2. Materials & Methods

Six human ovarian follicles were obtained from women having

spontaneous regular menstrual cycles. These women were healthy and were hospitalized for abdominal tubal ligations. They all had a curettage to obtain endometrial tissue for endometrial dating. Their ages ranged from 24 to 36 and they all had a history of normal pregnancies. Their children's ages ranged from 2 to 17 years.

The ovarian follicles were collected, transported, dissected, incubated, harvested and the incubation media collected at 24 hour intervals for a period of 3 days. The culture media was assayed for testosterone, oestradiol-17 β and progesterone. All of these procedures were carried out as described in Chapter 2 of this thesis.

Dr. Gillian Bryant, from the Department of Anatomy and Reproductive Biology, University of Hawaii, Honolulu, Hawaii kindly determined the relaxin released by these follicles into the culture media. The radioimmunoassay procedure used was the one described by Bryant (1972) and Bryant et al., (1975).

These six follicles were removed at specific times within the menstrual cycle. At the time of explantation, particular care was taken to remove the largest visible follicle. Particular attention was paid to the presence or absence of a corpus luteum and to explantation of the follicle on the ipsilateral or contralateral side to the corpus luteum. Two follicles studied were explanted during the early follicular phase (days 1-7/28), two follicles from the late follicular phase (days 8-14/28), and two follicles during the late luteal phase (day 25/28), one from the ovary containing the corpus luteum and one from the contralateral ovary.

7.3. Results

This series of preliminary data was too small for any statistical evaluation to be performed. However definite trends were apparent.

The trends in steroid release by these follicles fitted the patterns determined and illustrated in Chapter 2 (except for the abnormally low progesterone release by the 11.5 mm diameter late follicular phase follicle).

The amount of relaxin released by the follicle (6) removed from the ovary containing the corpus luteum on day 25 of the late luteal phase was high in comparison to the relaxin released from the follicle (5) removed from the ovary which did not contain the corpus luteum; also explanted in the late luteal phase of the menstrual cycle. Relaxin release by follicular phase follicles was also low.

Follicle	Day of explanation	Diameter	Day in culture	Steroid & relaxin levels released by each follicle, expressed as ng per mg of follicular tissue per 24 hours			
				Testosterone	Oestradiol-17 β	Progesterone	Relaxin
1 (early follicular)	4/28	6.3	1	113	19	10	3.8
			2	163	16	7	1.2
			3	82	12	7	0.8
2 (early follicular)	7/28	6.1	1	23	30	14	4.3
			2	52	34	21	1.4
			3	51	24	63	3.8
3 (late follicular)	12/28	5.4	1	19	27	89	1.8
			2	17	30	147	1.2
			3	6	27	97	0.8
4 (late follicular)	13/28	11.5	1	22	21	4	4.8
			2	30	33	6	1.9
			3	12	30	9	8.4
5 (from ovary without C.L.)	25/28	6.0	1	16	7	11	8.9
			2	30	31	9	4.1
			3	19	18	29	2.4
6 (from ovary with C.L.)	25/28	7.3	1	31	3	13	3.1
			2	36	14	20	15.1
			3	4	9	22	24.7

7.4. Discussion

Some valuable but limited information can be gained from this preliminary study. It has been demonstrated that intact human Graafian follicles, invitro, release variable amounts of relaxin into their surrounding culture medium.

It has been shown that relaxin levels in the peripheral blood of human females was, "high over the period of menstruation, its concentration then decreased until the middle of the follicular phase when a peak equal in amplitude to the menstrual peak occurred. Relaxin immunoactivity remained fairly constant throughout the preovulatory LH surge; however as the LH levels dropped, relaxin immunoactivity increased reaching maximal levels for the whole cycle. Relaxin immunoactivity then increased gradually over the luteal phase and reached a peak when progesterone levels were declining; it then dropped to low

levels until menses ensued", (Bryant, Panter and Stelmasiak, 1975). This pattern of peripheral blood relaxin activity tended to be followed to some extent by isolated follicle relaxin release, invitro. It was noted by Bryant et al., (1975) that it was not possible from their experiments to determine whether the relaxin was ovarian or uterine in origin. There is evidence to suggest that there is both a uterine and ovarian source of relaxin. Hughesdon (1976) has suggested that the endometrial K cells (a possible derivative from endometrial stroma) were plentiful during the premenstrual phase and probably contained relaxin. He did not give evidence of support this assumption. From the observations noted in this work it can be said that an ovarian source of relaxin was determined and furthermore at least some of it was of follicular origin.

It has been noted that relaxin levels rose as progesterone levels fell just prior to parturition (Sherwood, Chang, Bevier and Dziuk, 1975) in the pig, and in the human, luteal phase levels of blood relaxin also rose as progesterone levels fell just prior to menstruation. This reciprocal relationship between relaxin and progesterone activity suggested that a relationship between these substances may exist. Other evidence to support this thesis came from the work of Belt et al., (1971) who found from a histological and bioassay study, evidence that relaxin may arise from granulosa cells of the corpus luteum. The granulosa cells of the corpus luteum and follicle are almost certainly responsible for most of the progesterone production. The observation that the follicle explanted from the late luteal ovary which contained the corpus luteum, released the highest levels of relaxin in culture, and indicated that 'terminal' corpus luteum and associated luteal phase follicles as having high relaxin activity.

The physiological effects of high levels of relaxin released by the late luteal phase follicle could only be speculative at this stage. These observations indicated that relaxin may play a role in corpus luteum and follicular regression. Evidence has been put forward indicating that prostaglandin $F_2\alpha$ ($PGF_2\alpha$) may have a direct biochemical action in contributing to luteal regression (Henderson and McNatty, 1975; McNatty, Henderson and Sawers, 1975). They found that PGE_2 markedly stimulated the production of progesterone by human granulosa cells, whereas $PGF_2\alpha$ either alone or in combination with LH and FSH inhibited the production of progesterone. Their studies suggested

that $\text{PGF}_2\alpha$ initiated functional luteolysis, by having a direct action on the adenylate cyclase system within the granulosa cell. This action in turn blocked the synthesis of c-AMP which was necessary for eventual progesterone synthesis. Henderson et al., (1975) have proposed that $\text{PGF}_2\alpha$ acts on the intracellular 'coupling unit' to prevent the signal following LH induced receptor activity of the cell membrane, from induction of the catalytic site for adenylyl cyclase activation, thereby preventing the conversion of ATP to c-AMP. Data obtained in the ewe by Baird and Scaramuzzi (1975) showed that increased levels of $\text{PGF}_2\alpha$ from the ewe uterus was associated with a fall in progesterone and structural luteolysis. The cause of this final irreversible morphological deterioration of the corpus luteum was felt to be related to activation of lysosomes, absence of secretory granules and the appearance of autophagocytic bodies in the luteal cells (Gemzell, Stacey and Thornburn, 1976). However in the human, although $\text{PGF}_2\alpha$ inhibited the secretion of progesterone by luteal cells invitro, it did not cause structural damage (Henderson et al., 1975). A prostaglandin induced vascular mechanism contributing of human luteolysis in the human has not been demonstrated.

The findings in this study, in association with the observations made by other workers indicated that relaxin released by the late luteal phase follicle may reflect similar activity by the corpus luteum granulosa cells. Relaxin may play a role in functional luteolysis since its rise was associated with a fall in progesterone production. The rise in peripheral blood relaxin levels shortly after the pre-ovulatory LH peak may be associated with the breakdown of the follicular basement membrane (Bryant et al., 1975), theca interna and externa collagen tissue. It has been shown that relaxin was associated with the breakdown of collagenous tissue in the symphysis pubis and uterine cervix of the rat (Steinetz et al., 1976). The changes in collagen patterns within the Graafian follicle have been studied and are outlined in the following chapter.

7.5. Summary

It has been demonstrated that follicles explanted in both the follicular and luteal phase of the menstrual cycle were able to release relaxin in organ culture. Maximum release of relaxin occurred from those follicles explanted during the luteal phase and in particular from the follicles removed from the ovary containing the corpus luteum; a time when progesterone activity in the corpus luteum and Graafian follicle was declining. This hormone may be involved in both functional and morphological regression of both the late luteal phase corpus luteum and ovarian follicle. There is increasing evidence to implicate relaxin as a mediator of follicle basement membrane and theca interna and externa and collagenase activity. This role also indicates that relaxin may play an important part in facilitating ovulation by contributing to the breakdown of collagen at the point of ovum release by the ovulating follicle.

8. THE DISTRIBUTION OF COLLAGENOUS CONNECTIVE TISSUE IN THE HUMAN GRAAFIAN FOLLICLE

8.1. Introduction

There is increasing evidence that the layer of theca cells within ovarian follicles of mammals normally contains collagen. It has been proposed that the mechanism of ovulation required the disruption of collagenous connective tissue in the follicle wall (Espey, 1976). At least two "collagenolytic" enzymes have been detected in appreciable amounts in mature Graafian follicles of the rabbit. It was concluded that these enzymes could be active in the digestion of the follicle wall during ovulation (Espey and Coons, 1976).

An attempt has been made to determine if collagenous tissue existed within the theca interna of human follicles and also, to see if the collagen patterns changed in the theca externa and interna and basement membrane in relation to follicular size and the time of oophorectomy during the menstrual cycle.

8.2. Materials & Methods

A series of normal ovaries obtained at various stages throughout the menstrual cycle were processed histologically and stained specifically for the localization of collagen patterns within small and large Graafian follicles, ovarian stroma and corpora lutea.

8.2.1. van Gieson's method for collagen staining

The method used was an accepted method using van Gieson's stain (Culling, 1974), and kindly performed by Mr. Roy Ellis, Laboratory Manager, Department of Cytology and Gynaecological Pathology, The Queen Elizabeth Hospital, Adelaide.

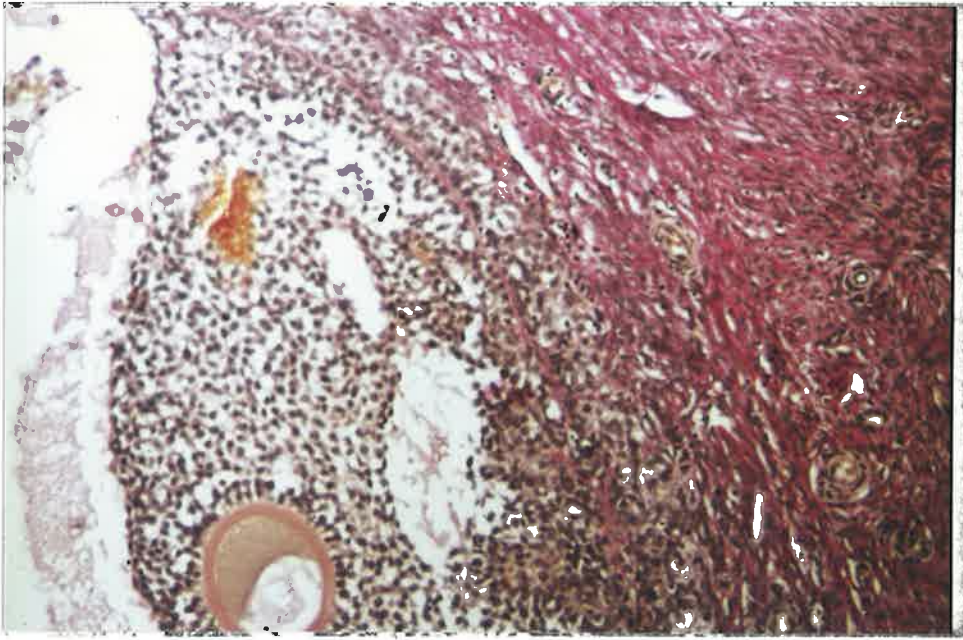
van Gieson's Stain

- Saturated aqueous solution of picric acid
(approximately 1 per cent) 100 ml
- 1 per cent acid fuchsin 10 ml

The above quantities may be prepared and kept as a stock solution, but it is better to use a freshly prepared solution containing 5 ml of saturated aqueous solution of picric acid and 0.75 ml of 1 per cent acid fuchsin which gives more precise and sharp staining.

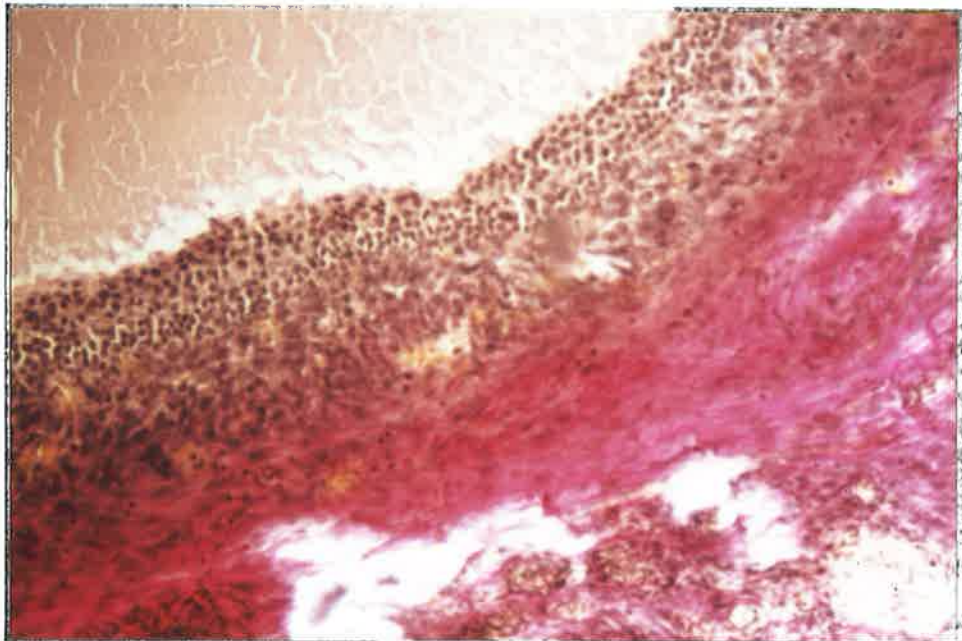
Staining Technique (van Gieson)

- (1) Bring section to water.



Photomicrograph 28 (x250)

The basement membrane is prominent and stains in an identical fashion to the fine network of collagen dispersed throughout the theca interna layer. The denser circumferentially arranged collagen can be seen in the theca externa.



Photomicrograph 29 (x250)

A denser layer of collagenous tissue can be seen surrounding the follicle which is probably due to a condensation effect by the growing follicle.

- (2) Stain with celestin blue for 5 minutes, wash and add Harris's haematoxylin for 3 minutes.
- (3) Blue in tap-water.
- (4) Differentiate in 1 per cent acid alcohol.
- (5) Blue in tap-water.
- (6) Place the slide on the rack and flood with van Gieson's stain, leave for 3 minutes.
- (7) Rinse rapidly in tap-water to differentiate the fuchsin.
- (8) Dehydrate in 90 per cent and absolute alcohol which a few drops of saturated picric acid in alcohol have been added, rinse quickly in fresh absolute alcohol, blot and clear in xylol (picric acid is removed rapidly by alcohol, and the addition of picric acid to the dehydrating bath prevents this).
- (9) Mount in acid balsam (to preserve the counterstain) or D.P.X.

Results

Nuclei	Blue black
Collagen	Bright red
Cytoplasm, muscle, red blood cells	Yellow

8.3. Results

Collagen was demonstrated in the theca interna of follicles ranging from 2 to 12 mm in diameter and was present in ovaries explanted during both the follicular and luteal phase of the menstrual cycle. The collagen fibres appeared as very fine elongated processes which were more densely arranged near the inner margin of the theca interna layer. They appeared to extend between the individual theca interna cells and reached the basement membrane.

See photomicrograph 28.

The basement membrane took up the van Gieson's stain and stained in an identical fashion to the collagen present amongst the theca interna cells. The zona pellucida surrounding the ovum was more lightly stained which indicated that it was of a different composition (see photomicrograph 28).

Collagen was distributed in a circumferential pattern around follicles. The density of collagen increased as the follicle increased in size.

See photomicrograph 29.

It appeared probable that the increased density of collagen around the larger follicles was due to a condensation effect of the growing follicle on the surrounding theca externa collagen/connective tissue. There was no evidence of any remnants of the basement membrane in early or late corpora lutea. There was abundant collagen in the immediate stroma surrounding corpora lutea.

8.4. Discussion

A selective histological staining method was used, which indicated that collagen connective tissue existed in follicular and luteal human ovarian follicles. It was thought that the rat follicle was devoid of collagen, (Parr, 1974) but it was later shown that collagen did exist in rat follicles (Espey, 1976).

Since theca interna and basement membrane connective tissue was present in follicles and absent in corpora lutea something must have occurred during the periovular period to destroy the connective tissue. Particular attention was not applied to the very late or premenstrual luteal follicles with respect to collagen patterns. The rise in relaxin levels about the time of ovulation (Bryant et al., 1975), and increased collagenase activity in large follicles (Espey et al., 1976) could well be more than a coincidental finding. It is tempting to speculate that there may be an association between high relaxin levels, collagenase activity and $\text{PGF}_2\alpha$ in order to facilitate physiological and morphological regression of the corpus luteum.

8.5. Summary

Collagen was demonstrated in the theca interna but was more marked in the theca externa of follicles from 2 to 12 mm in diameter. Collagen deposition was more abundant as the follicles became larger and was also present in luteal phase follicles. Collagen deposition was dispersed in the theca cells of the corpus luteum but was present in abundance in the surrounding stroma. The basement membrane lying between the theca and granulosa cells of follicles was prominent and stained identically to that of collagen. Evidence of the basement membrane or its remnants were not seen in corpora lutea. The circumferential pattern of collagen surrounding follicles probably appeared more prominent as the follicles grew due to a condensation effect on the surrounding connective tissue.

9. BLOOD PROLACTIN LEVELS BEFORE, DURING AND FOLLOWING SURGERY:
FOLLICULAR STEROID ACTIVITY DURING INCUBATION OF WHOLE
FOLLICLES WITH FSH AND PROLACTIN

9.1. Introduction

Blood prolactin levels have been shown to vary in relation to anaesthetic stress in rats (Simone1, Brooks & Welsch, 1975), in disorders of ovulation and menstruation (Pepperell, Bright and Smith, 1977) and in women on combined steroid oral contraception and in the postmenopausal period. A great deal of research has been done in order to develop an understanding with regard to prolactin physiology at a hypothalamic-pituitary level (Archer, 1977; Turkington, 1973). The physiological action of prolactin at an intraovarian level is still uncertain.

Many forms of stress appear to be associated with an elevation of blood prolactin and it was therefore decided to measure blood prolactin changes in relation to anaesthetic and surgical stress. An attempt was also made to assess the effect of incubating human ovarian follicles with prolactin in relation to their steroid activity.

9.2. Methodology

9.2.1. Assessment of peripheral venous prolactin before, during and after surgery in relation to follicular fluid prolactin, sodium and potassium concentration and osmolality during surgery

Women within the reproductive age group who were having spontaneous regular menstrual cycles had peripheral venous blood collected the day before, a half an hour after the commencement of surgery and twenty four hours following surgery. Six of these women had a tubal ligation and two an abdominal hysterectomy for fibroid uteri. Follicular fluid was aspirated under direct vision from the largest follicles present in the ovaries. An estimation of follicular size was recorded. Particular note was made of the date of the last menstrual period and an endometrial curette was performed for endometrial dating.

Radioimmunoassays used for the assessment of prolactin, LH & FSH

Mr. Kevin Crawshaw (Scientific Officer, Department of Radioimmunoassay Procedures, Queen Elizabeth Hospital, Adelaide), kindly performed estimations of prolactin, LH and FSH on both the peripheral venous blood and follicular fluid. Estimations of follicular fluid osmolality, sodium and potassium concentrations were performed in the Department of Clinical Chemistry, Queen Elizabeth Hospital, Adelaide.

The anaesthetic procedure consisted of a premedication one hour prior to surgery (usually 100 mg Pethidine I.M. or 20 mg of Omnopon I.M.). Induction of anaesthesia with I.V. Thiopentone and maintained with a combination of Nitrous Oxide and Halothane was used. A muscle relaxant was always used to facilitate the abdominal surgical procedure.

9.2.2. Steroid activity of human ovarian follicles during incubation with FSH and prolactin

Materials

FSH - Human pituitary gland extract (CSL). See 3.2.1. for details.

Prolactin - Human prolactin (200 ng/ampoule). Calbiochem, La Jolla, California (Cat. no. 869058).

As there may have been viable pathogens in the freeze dried preparation of prolactin, the 200 ng of powder was dissolved in 1 ml of culture medium and filtered through an 0.45 u millipore filter.

Methodology

Three late proliferative phase follicles were selected and set up in culture media as described in Chapter 2.

Follicle 1 - explanted on day 8/28 - diameter 6 mm

Follicle 2 - explanted on day 10/28 - diameter 7 mm

Follicle 3 - explanted on day 11/28 - diameter 6 mm

For the first 24 hours in culture (Day 1), no FSH or prolactin was added, on Day 2, 20 iu of FSH per ml of culture media was added, on Day 3 and 4, 20 iu of FSH + 200 ng of prolactin was added to the culture media.

Results

See Tables 1 and 2.

Serum prolactin was significantly raised during surgery when compared to values determined on the day prior to surgery (** $p < 0.025$). Prolactin levels were still raised on the day following surgery but were significantly lower than the values found during surgery ($0.1 > p > 0.05$).

There did not appear to be a differential pattern of prolactin blood levels in relation to the timing of surgery during the menstrual cycle. Although the number of follicles was small it could be said that there was no apparent trend in follicular fluid prolactin levels in relation

Table 1. The effect of anaesthetic and surgical stress on blood prolactin levels. Follicular fluid levels of prolactin, sodium, potassium and osmality values during the surgical procedures.

Patient/ follicle number	BLOOD Prolactin levels (ng/ml of serum)			Follicular diameter	Prolactin	FOLLICULAR FLUID			STAGE OF CYCLE
	Day prior to surgery	Surgery for ½ hour	Day after surgery			Sodium	Potassium	Osmality	
1	9	170	37	5.5	7.8	156	5.2	280	Early foll
2	3.3	62	-	6.0	11	137	4.4	308	Late foll
3	7.0	130	42	8.4	5.0	142	4.5	340	Late foll
4	5.0	92	-	7.0	7.2	137	4.3	340	Late foll
5	6.0	52	27	11.5	7.5	139	4.4	276	Late foll
6	13	112	59	6.0	32	155	5.2	310	Late luteal
Mean	*** 7.2	78	* 41						
SEM	± 1.4	± 24	± 7						

Normal Ranges - Serum Prolactin 2.5 - 10 ng/ml (for ovulating premenopausal women)
 Serum LH 5 - 26 mu/ml (excluding the preovular LH surge values)
 Serum FSH 5 - 20 mu/ml
 Serum Osmality 275 - 295 milliosmols/Kg of water
 Serum Sodium 137 - 144 m.mol/litre
 Serum Potassium 3.5 - 5.4 m.mol/litre

* denotes p < 0.1
 *** denotes p < 0.025

Table 2. Steroid activity of human ovarian follicles during incubation with FSH and prolactin

Steroid released expressed as ng per mg of follicular tissue per 24 hours	Follicle	Days in organ culture			
		1	2	3	4
		CONTROL	+ FSH	+ FSH + PROLACTIN	+ FSH + PROLACTIN
TESTOSTERONE	1	23	145	106	76
	2	44	160	20	4
	3	28	-	28	3
OESTRADIOL-17 β	1	27	91	50	18
	2	28	51	14	4
	3	20	-	-	3
PROGESTERONE	1	4	164	86	21
	2	22	67	16	7
	3	19	40	11	6

to follicular size or stage of the menstrual cycle at the time of surgery (apart from a high value recorded from a luteal phase follicle which may or may not be significant depending upon further evaluation). Sodium and potassium values appeared to be higher in the early proliferative and luteal phase follicular fluid but the observations were too small to permit interpretation. Follicular fluid osmolality was quite variable (276-340) but a pattern of osmolality variation was not apparent in this small preliminary study. (See Table 1).

There did not appear to be a significant change in blood levels of FSH or LH prior to, during or following surgery. The range of values for FSH was 2.1 - 14 $\mu\text{u/ml}$ ($n = 6$) and for LH was 7.9 - 25.4 ($n=6$).

The incubation of late follicular phase follicles with FSH was followed by a predictable increased release of testosterone, oestrogen and progesterone, invitro (see Chapter 3). The addition of high levels of prolactin to the culture media tended to suppress steroidogenesis. However the numbers are too small to say if this observation was due to prolactin or due to the predicted decline in steroid activity with time in culture. (See Table 2).

9.3. Discussion

A significant rise in peripheral venous blood prolactin occurred as a result of the stress of anticipation of surgery, induction of anaesthesia or surgery or a combination of all three factors. The effects of ether anaesthesia on plasma prolactin sampling has been investigated by Simonel, Brooks and Welsch (1975). They found that rats subjected to ether anaesthesia had a significant elevation of their blood prolactin levels within two minutes of exposure to ether. In order to more accurately determine the individual factors which contributed to the elevated prolactin levels, very frequent blood samples would have to be taken prior to the anaesthesia, during the induction-surgery interval, during surgery and following surgery. All that can therefore be said was that the stress of anticipation, anaesthesia and surgery may all contribute to an elevated prolactin level. These results demonstrate that great care must be taken to exclude stress factors when an attempt is made to evaluate blood prolactin values. Pepperell, Bright and Smith (1977) found that blood prolactin levels did not vary significantly throughout the menstrual cycle. This observation indicated that the stress of blood sampling was not a significant factor, contributing to a variation in prolactin release. Despite the high levels of circulating prolactin

during surgery, the follicular fluid levels appeared to be independent of these blood changes at the time of aspiration.

Prolactin plays a varied role in the reproductive processes of many species. It has been shown to influence the osmoregulation in fish which migrate between salt and fresh water when spawning (Yuen, Keye and Jaffe, 1973). As a result of these observations a preliminary attempt was made to assess sodium, potassium and osmality values in follicular fluid. Possibly because of insufficient data no obvious trend in these parameters was apparent.

The incubation of late follicular phase follicles with FSH was followed by a predictable increased release of testosterone, oestrogen and progesterone, invitro. However the addition of high (unphysiological) concentrations of prolactin to the culture media did not appear to augment follicular steroid activity, and possibly tended to suppress steroidogenesis. It has been noted that large steroid active pre-ovular follicles contained low physiological levels of prolactin (McNatty, Hunter, McNeilly and Sawers, 1975). In another study, McNatty, et al., (1974) were able to show that the production of progesterone by human granulosa cells, invitro, required low physiological concentrations of prolactin, whereas high concentrations were inhibitory. The invitro, studies reported in this study tend to support these observations.

9.4. Summary

The combination of anaesthesia and surgery was associated with a significant elevation of blood prolactin levels. However follicular fluid prolactin levels obtained during surgery appeared to be independent of blood levels over the time interval studied. These initial studies were unable to implicate prolactin as an osmoregulator in the ovarian follicle. The incubation of late follicular phase follicles with high unphysiological levels of prolactin in the presence of FSH tended to inhibit follicular steroid release.

10. THE RELATIONSHIP BETWEEN TESTOSTERONE AND Δ^4 - ANDROSTENEDIONE
RELEASE BY HUMAN OVARIAN FOLLICLES INVITRO

A radioimmunoassay on culture medium for the estimation of androstenedione was developed in our laboratory when this thesis was almost completed. It was considered important to determine the release of androstenedione by human ovarian follicles in culture, because a great deal of evidence has indicated that this may be the major androgen produced by the theca interna cells.

Preliminary results have shown that human ovarian follicles in organ culture released five to six times as much androstenedione as testosterone. However it was demonstrated that a predictable linear, qualitative relationship existed between testosterone and androstenedione release, for follicles explanted throughout the human reproductive cycle. As testosterone release increased from follicles invitro, so did androstenedione.

See graph 1

More investigation is being undertaken in order to determine a better understanding of the relationship between these two androgens which are released by human Graafian follicles, invitro.

GRAPH 1

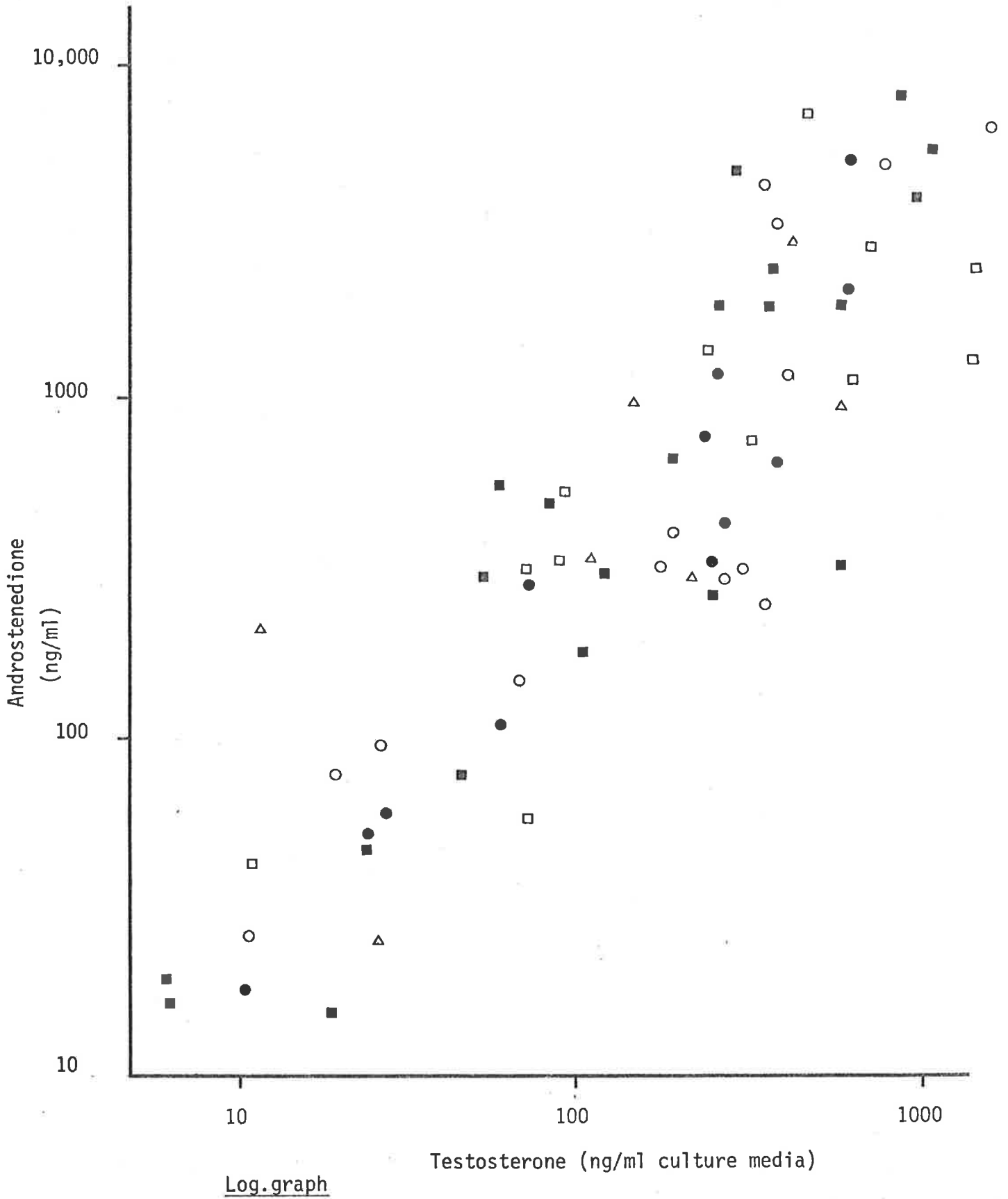
Logarithmic plot of androstenedione release in relation to testosterone release expressed as ng of steroid per ml of culture media, of human ovarian follicles removed at the following stages of the human reproductive cycle:-

- △ Early follicular phase (days 1-7/28)
- Late follicular phase (days 8-14/28)
- Luteal phase (days 15-28/28)
- Post partum (days 1-5 post parturition)
- Small follicles from a patient with polycystic ovarian disease and signs of virilism

It can be seen that there is a linear qualitative relationship between androstenedione and testosterone release by follicles removed from patients with variable endocrine features.

GRAPH 1

Relationship between Δ^4 -androstenedione and testosterone release by human ovarian follicles in organ culture



APPENDIX

EARLY FOLLICULAR PHASE EXPLANTATION (DAYS 1-7)

Unstimulated Follicles in Organ Culture

CODE	DAY OF CYCLE	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
			1	2	3	4	1	2	3	4	1	2	3	4				
71-1	6	6.6	++	2	+	1	23	52	51	18	30	34	24	9	14	21	63	15
44-3	4	5.0	+++	3	+	1	35	72	24						13	15	13	
44-4	4	6.4	+++	3	+	1	23	121	68	19	6	4	6	5	7	6	9	
44-5	4	6.3	+++	3	-	0	19	168	82	30	2	19	16	12	10	7	7	
33-2	5	3.2	+	1	-	0	17	15	11		2	4	2		10			
85-1	7	4.8	++	2	+	1	16	25	16	10	28	37	30	20	2	2	2	2
85-2	7	5.4	++	2	+	1	28	29	21	25	16	18	19	2	1	1	1	1
<u>Statistical analysis</u>																		
\bar{X}	5	5.4	2.2		0.7		23	70	41	20	14	19	15	10	7	9	16	6
v	1.9	1.4	0.6		0.7		45	3209	782	57	162	220	130	48	33	61	553	61
SD	1.4	1.3	0.8		0.5		7	56	28	8	13	15	11	7	6	8	23	8
SEM	0.5	0.5	0.3		0.2		3	21	11	3	5	6	5	3	2	3	9	4
n	7	7	7		7		7	7	7	5	6	6	6	5	7	6	6	3

LATE FOLLICULAR PHASE EXPLANTATION (DAYS 8-14)

Unstimulated Follicles in Organ Culture

CODE	DAY OF CYCLE	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
							1	2	3	4	1	2	3	4	1	2	3	4
70-1	10	5.4	++	2	++	2	19	17	6	11	7	27	30	27	89	147	97	27
68-1	13	11.5	++	2	+++	3		22	30	12	44	21	33	30	26		27	47
63-1	10	7.0	++	2	++	2	23				27	36	50	18		164		86
62-1	11	8.4	++	2	++	2	14	68	29	12	15	16	28	15	26	46	40	50
52-1	14	7.5	++++	4	+++	3	23	53	6	26	40	41	24	11				32
83-1	9	10	++++	4	++	2	15	17	38	20		41	68	37				
<u>Statistical analysis</u>																		
\bar{X}	11	8.5	2.7		2.3		19	36	22	16	30	30	39	23	47	119	55	48
v	4	4.7	1.1		0.3		18	556	220	43	277	113	284	98	1344	4049	1386	536
SD	1.9	2.1	1.0		0.5		4	24	15	7	17	11	17	10	37	64	37	23
SEM	0.8	0.8	0.4		0.2		2	10	7	3	8	4	7	4	21	37	21	10
n	6	6	6		6		5	5	5	5	4	6	6	6	3	3	3	5

LUTEAL PHASE EXPLANTATION (DAYS 15-28)

Unstimulated Follicles in Organ Culture

CODE	DAY OF CYCLE	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
							1	2	3	4	1	2	3	4	1	2	3	4
69-1	22	6.0	+	1	+	1	16	30	19	9	7	31	18	11	11	9	29	13
51-1	28	5.3	+	1	+	1	10	13	8	6		9	10	7				
46-1	18	7.6					22	29	20			9	4	2	14	22	24	12
46-2	18	7.3	++	2	++	2	31	36	41		3	14	9	6	13	20	22	28
40-1	24	5.5	++++	4	+++	3		70	30		5	10	5	3	23	19	20	
36-1	16	5.5					28	37	25	18	24	20	13	5				
34-1	15	4.0					40	23	9	4			7	1				
<u>Statistical analysis</u>																		
\bar{X}	19.6	5.5	2.0	1.8	21	34	22	9	10	15	9	5	15	18	24	18		
v	21	2.3	2.0	0.9	155	319	135	38	93	69	27	11	28	34	15	80		
SD	4.6	1.5	1.4	0.9	13	18	12	6	10	8	5	3	5	6	4	9		
SEM	1.6	0.5	0.7	0.5	5.1	6.7	4	3	4.8	3	1.8	1.2	2.7	2.9	1.9	5.1		
n	8	8	4	4	6	7	7	4	4	6	7	7	4	4	4	3		

LATE FOLLICULAR PHASE EXPLANTATION (DAYS 8-14)

Incubation of Follicles with FSH

CODE	DAY OF CYCLE	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
			1	2	3	4	1	2	3	4	1	2	3	4				
70-2	12	5.5	+	1	+	1	15	54	28	13	12	139	26	12	10	102	39	48
68-1	13	11.5	++	2	+++	3	14	64	14	12			33	30				47
63-1	10	7.0	++	2	+++	3	23	145	106	76	27	91	50	18	25	164	203	86
62-2	11	5.6	++	2	++	2	44	160	20		28	51	14		22	67		
53-2	6	5.7	+	1	++	2	30	32	22	10		354	109	64		253	196	89
<u>Statistical analysis</u>																		
\bar{X}	10	7.1		1.6		2.2	25	91	38	28	22	156	46	31	19	147	146	68
v	7.3	6.5		0.3		0.7	153	3314	1470	1036	80	18394	1394	540	63	6649	8599	535
SD	2.7	2.6		0.5		0.8	12	57	38	32	9	135	37	23	8	82	92	23
SEM	1.2	1.1		0.2		0.4	6	25	17	16	5	67	17	11	5	41	53	12
n	5	5		5		5	5	5	5	4	3	4	5	4	3	4	3	4

EARLY FOLLICULAR PHASE EXPLANTATION (DAYS 1-7)

Incubation of Follicles with HCG (20 iu/ml of culture medium)

CODE	DAY OF CYCLE	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				GESTROGEN				PROGESTERONE			
							1	2	3	4	1	2	3	4	1	2	3	4
61-1	2	5.0	+++	3	+	1	41	108	64	16	7	37	16	6	104	125	67	31
60-1	2	3.1	+	1	+	1	30								55	118	55	18
44-2	4	5.5	+++	3	+++	3	96	158	69	32	12	14	12	13	26	144	23	10
43-1	7	4.8	+++	3	+++	3	24	24	11		20	21	7	2	22	30	56	38
<u>Statistical analysis</u>																		
\bar{X}	4	4.6	2.5		2.0		48	97	48	24	13	24	10	7	52	104	50	22
v	5.6	1.2	1.0		1.3		1084	4585	1033	128	43	139	60	31	1429	2570	359	278
SD	2.3	1.1	1.1		1.2		32	68	32	11	7	12	8	6	37	50	19	17
SEM	1.2	0.5	0.5		0.5		16	39	18	8	3	6	4	3	18	25	9	8
n	4	4	4		4		4	3	3	2	3	3	3	3	4	4	4	4

LATE FOLLICULAR PHASE EXPLANTATION (DAYS 8-14)
Incubation of follicles with HCG (20 iu/ml of culture medium)

CODE	DAY OF CYCLE	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
			1	2	3	4	1	2	3	4	1	2	3	4				
57-1	9	7.0	+	1	++	2	86	78	28		4	4	3		162	81	55	
48-1	13	6.0	++	2	++	2	15	42	28	20	15	17	6	2	30	72	49	29
48-3	13	5.0	++	2	++	2					12	13	8	6	51	167		
45-3	10	6.2	++++	4	+++	3	87	136	49			12			33	46	43	45
<u>Statistical analysis</u>																		
\bar{X}	11	6.1	2.3		2.3		63	85	35	(20)	7	12	6	4	69	92	49	37
v	4.3	0.68	1.5		0.3		1704	2249	147		49	20	6	8	3930	2753	36	128
SD	2.1	0.82	1.2		0.5		41	47	12		7	5	3	3	62	52	6	11
SEM	1.0	0.41	0.6		0.3		23	27	7		4	3	1	2	31	26	3	8
n	4	4	4		4		3	3	3	1	3	4	3	2	4	4	3	2

LUTEAL PHASE EXPLANTATION (DAYS 15-28)

Incubation of Follicles with FSH

CODE	DAY OF CYCLE	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
							1	2	3	4	1	2	3	4	1	2	3	4
69-3	22	5.2	+++	3	+	1	22	50	14	3	31	43	11	2	47	173	47	41
55-2	24	5.8	+	1	++	2	5	4	64		65	55	20		234	214	106	
57-1	15	5.0	+	1	+++	3			28		6	4	3		162	81	55	
<u>Statistical analysis</u>																		
\bar{x}	20	5.3	1.7		2.0		14	27	35		34	34	11		148	156	69	
v	22	0.2	1.3		1		144	1058	665		877	711	72		8896	4639	1024	
SD	4.7	0.4	1.2		1		12	33	26		30	27	9		94	68	32	
SEM	2.7	0.2	0.7		0.6		8	23	15		17	15	5		54	39	18	
n	3	3	3		3		2	2	3		3	3	3		3	3	3	

LUTEAL PHASE EXPLANTATION (DAYS 15-28)

Incubation of Follicles with HCG

CODE	DAY OF CYCLE	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
							1	2	3	4	1	2	3	4	1	2	3	4
69-2	22	4.8	+++	3	+++	3	22	41	15	2	43		31	12		74	140	
51-2	27	5.2	+++	3	+++	3	52	29	10	5	3	9	8	3				
40-2	24	5.2	+++	3	++	2	36	21	8						45	42	39	
40-3	24	5.1	++++	4	+++++	5	68	85	23	8	8	2	1		61	42	44	
39-1	19	4.7	++++	4	+++++	5	103	146	54		40	46	26		129		140	
<u>Statistical analysis</u>																		
\bar{x}	<u>23</u>	<u>5</u>	<u>3.4</u>		<u>3.6</u>		<u>56</u>	<u>64</u>	<u>22</u>	<u>5</u>	<u>24</u>	<u>19</u>	<u>22</u>	(8)	<u>78</u>	<u>53</u>	<u>91</u>	
v	8.7	0.1	0.3		1.8		981	2692	354	9	438	559	146	40	1989	341	3238	
SD	2.9	0.2	0.5		1.3		31	52	19	3	21	24	12	6	44	18	56	
SEM	1.3	0.1	0.2		0.6		14	23	8	2	10	13	7	4	25	11	28	
n	5	5	5		5		5	5	5	3	4	3	4	2	3	3	4	

POST PARTUM EXPLANTATION (DAYS 2-5 POST DELIVERY)

Unstimulated Follicles in Organ Culture

CODE	DAY OF CYCLE-POST PARTUM	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
							1	2	3	4	1	2	3	4	1	2	3	4
67-1	5	3.3	+	1	+	1	31	36	12	7		58	18	12	62	13	11	3
66-1	3	4.9					69				72			27				
66-2	3	4.3					68				60			24				
66-3	3	5.3					70				31			18				
66-4	3	4.3					63				48			27				
65-1	3	3.5	+	1	+	1	131	82	28		81		31	11	29	27	21	
64-2	2	4.4	+	1	+	1	84	57	53		16	31	20		36	42	36	
58-2	2	3.2	+	1	+	1	89	58	17	3	8	10	6	3	43	59	28	22
<u>Statistical analysis</u>																		
\bar{X}	3	4.2	1	1	76	58	28	5	45	33	19	9	27	35	27	(13)		
v	0.8	0.6	0	0	800	353	334	8	777	579	105	24	345	391	164	180		
SD	0.9	0.8	0	0	28	19	18	3	28	24	10	5	19	20	13	13		
SEM	0.3	0.3	0	0	10	9	9	2	10	14	5	3	7	10	6	10		
n	8	8	4	4	8	4	4	2	7	3	4	3	8	4	4	2		

POST PARTUM EXPLANTATION (DAYS 2-4 POST DELIVERY)

Incubation of Follicles with FSH

CODE	DAY OF CYCLE POST PARTUM	DIAMETER (mm)	3 β -HSD ACTIVITY IN				STEROID LEVELS FOR EACH DAY IN CULTURE (ng of steroid/mg of follicular tissue per 24 hours)											
			THECA		GRANULOSA		TESTOSTERONE				OESTROGEN				PROGESTERONE			
							1	2	3	4	1	2	3	4	1	2	3	4
58-1	2	4.4	+	1	+	1	88	79	81	36	32	16	8	4	145	53	56	40
58-3	2	3.0	+	1	+	1	84	77	43	34	5	3		20	11	9	12	
56-1	4	4.8	+	1	++	2	59	59	37	10	35	35	18	9	144	123	71	35
<u>Statistical analysis</u>																		
\bar{X}	2.7	4.1		1		1.3	72	72	54	27	24	18	13	7	103	62	45	29
v	1.3	0.9		0		0.3	247	121	569	209	273	259	50	13	5167	3201	1046	223
SD	1.2	0.9		0		0.9	16	11	24	14	17	16	7	4	72	56	32	15
SEM	0.7	0.5		0		0.3	9	6	14	8	9	9	5	3	41	32	18	8
n	3	3		3		3	3	3	3	3	3	3	2	2	3	3	3	3

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Units of Measurement

Kg	Kilogramme
g	gramme
mg	10^{-3} g
ug	10^{-6} g
ng	10^{-9} g
pg	10^{-12} g
i.u.	International units
mm	millimetre
c.p.m.	Counts per minute
r.p.m.	Revolutions per minute

Statistical

\bar{X}	Mean
S.D.	Standard deviation
SEM	Standard error of the mean
v	Variance
t	Students t-test