FOLLICULAR DEVELOPMENT AND GONADOTROPHINS

by

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

This study is dedicated to my parents,
who, with their continued encouragement
and assistance have made it all possible.

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Declaration

I declare that the experiments reported in this thesis were carried out by myself. Any assistance received from others is specifically acknowledged.

No part of this thesis has been submitted to any other University for any degree or diploma.

Timothy J. Weiss

PREFACE

Aspects of the work herein have been published as follows:-

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ABSTRACTS

Janson, P.O., Weiss, T.J., Amato, F., McIntosh, J.E.A., Seamark, R.F. (1976). Cyclic AMP release by sheep follicles following gonadotrophic stimulation. Int. Cong. Endocr. Hamburg. Ab. 173.

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SUMMARY

This thesis is concerned with a study carried out in sheep on the development of the ovarian antral follicle and, in particular, with the changing relationship between the follicle and pituitary gonadotrophins during the oestrous cycle.

1. Differences in cyclic AMP output between follicles of differing sizes indicated that the response of antral sheep follicles to gonadotrophins changes as the follicles developed. For this study, follicles of two size classes, 1 - 3 mm and 4 - 6 mm in diameter, were isolated from ovaries of sheep between Days 4 - 14 of the cycle and separated into their two major cellular components viz. theca and granulosa. The isolated tissue was incubated with FSH or hCG and the response to gonadotrophins was assessed by measuring changes in the production of cyclic AMP and steroids. It was found that the level of cyclic AMP in the theca of small follicles was increased following a 40 min incubation with hCG but not with FSH, whereas the granulosa cells of these follicles were unresponsive to hCG but responded to FSH. The small follicles produced predominantly testosterone in culture, with little or no detectable oestradiol. The appearance of oestrogen as a major secretory product of the larger follicles was associated with changes in the granulosa, as this tissue now responded to both LH and FSH in terms of cyclic AMP production.

Incubation of intact large follicles with LH or FSH increased the concentration of cyclic AMP in both follicular tissue and incubation medium. After 90 min incubation the level of cyclic AMP in the tissue treated with LH had reached a maximum while levels in the medium continued

to increase for up to 180 min after the addition of hormone. The considerable extracellular release of cyclic AMP suggests a possible means by which LH could influence the development of the granulosa viz. when LH acts on the theca, cyclic AMP release is stimulated and may then act as a local diffusion activator and stimulate the development of the granulosa.

2. Prior exposure of follicles to a specific gonadotrophin modified their subsequent response to that gonadotrophin, indicating that the response of a follicle in vivo to circulating gonadotrophins may not only depend on its stage of development but, at least in the short term, may also be regulated by previous changes in gonadotrophin levels. For this study, intact follicles were cultured overnight in the presence of hCG or FSH. The follicles were subsequently separated into theca and granulosa, and cyclic AMP levels were measured in the tissue after a second incubation with gonadotrophin. Preincubation with hCG resulted in a decrease in the ability of theca from large and small follicles, and granulosa from large follicles, to increase cyclic AMP levels in response to a second exposure to hCG. Similarly, preincubation of follicles with FSH suppressed the response of isolated granulosa from large and small follicles to a second exposure to FSH. A partial, but significant, heterologous inhibition was observed after preincubation of large follicles with both hCG and FSH, and in the theca of small follicles after preincubation with FSH. The results suggest that increases in the levels of LH and FSH may not only regulate the responsiveness of their own receptors, but may also influence the sensitivity of their target cells to the heterologous hormones.

In addition to the diminished cyclic AMP response of follicular tissue, androgen production by both small follicles and isolated theca and follicle wall from large follicles, was suppressed by pretreatment with hCG. The suppression by hCG, of the production of androgen by the theca was not overcome by incubating the tissue with dibutyryl cyclic AMP, suggesting that there are at least two points of regulation of steroidogenesis in the follicle, one pre- and one post-cyclic AMP production.

3. In order to determine whether the autoregulation of the follicular response to gonadotrophins was of physiological significance, a study was undertaken to examine the effect of hCG on cyclic AMP release from sheep ovaries in situ. There was considerable variation in the response of sheep ovaries to hCG given at different stages of the oestrous cycle. Injection of hCG resulted in a rise in cyclic AMP levels in the ovarian vein of ovaries containing a corpus luteum, and between Days 3 - 18 of the cycle. The ovaries of ewes examined at Days 1 and 2 of the cycle showed no response to hCG, whereas in two ewes at Day 3, hCG caused a slight response. In thirteen sheep examined between Days 5 - 18, hCG caused a marked increase in cyclic AMP and progesterone release. The results suggest that the corpus luteum is the major source of cyclic AMP in the ovarian venous effluent. The refractory period of the ovary following ovulation is probably due to both a desensitization of the LH receptor mechanism in the ovulatory follicle and to insufficient luteal tissue to measurably respond to hCG.

4. The possibility that a loss of responsiveness at the follicular level is the cause of post partum anoestrous in the cow was also investigated. Follicles were obtained from both post partum and cycling cows. Treatment of cycling cows with PMSG 24 h prior to ovariectomy increased oestrogen secretion of the isolated follicles during 18 h in culture. In contrast, follicles from post partum cows treated with PMSG did not exhibit the same increase in oestrogen output suggesting that the follicular steroidogenic response to gonadotrophic stimulation is suppressed in the post partum cow.

Pretreating the post partum animals with bromo-ergocryptine to suppress endogenous prolactin levels appeared to increase oestrogen production by isolated follicles. However, incubation of follicles from both post partum and cycling cows for 18 h with prolactin did not alter the steroid secretion of either group during the treatment period. Prolactin may affect the response of follicles to gonadotrophins as theca prepared from follicles pretreated with prolactin produced less cyclic AMP during an incubation with hCG than theca of untreated follicles. However, the physiological significance of this is unclear as there was no apparent difference in the cyclic AMP response of theca and granulosa from the follicles of post partum or cycling cows.

5. During the course of this study it became apparent that current method for distinguishing between atretic and non-atretic follicles, based on a histological assessment or an arbitrary assessment of steroid output were inadequate. An investigation was therefore made into the relationship between steroidogenesis and state of atresia of small antral sheep follicles. The steroid production of follicles classified as being

atretic or non-atretic on the basis of their morphology, was analyzed by means of discriminant analysis. The steroid output of individual atretic follicles was similar and characterized by a low to undetectable secretion of oestrogen. In contrast, secretion by non-atretic follicles varied considerably and was indicative of a continuum of physiological states. Histological examination of the follicles supports the proposal that the degree of atresia (pyknosis) can be related to changes in steroid output. It was concluded that the use of classification procedures, such as discriminant analysis, can provide an objective means of sorting follicles into atretic and non-atretic classes on the basis of their steroid output.

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The successful development of an ovarian follicle, culminating in ovulation, depends on a complex interplay between the hypothalamus, pituitary, and the different compartments of the ovary itself. Our understanding of the factors involved in the regulation of follicular development has been greatly enhanced by advances which followed the development of specific and sensitive assays for measuring levels of pituitary hormones, such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), and steroid hormones of ovarian origin, such as progesterone, oestrogen and testosterone. In addition, the recent advent of organ culture techniques has allowed an examination of the behaviour of individual ovarian tissues. This thesis is concerned with the growth and development of the antral follicle, and in particular, with the changing relationship between the follicle and pituitary gonadotrophins during the oestrous cycle.

The basis of our present understanding of follicular development has resulted largely from experiments with rodents, particularly rats and mice. However, in the studies reported in this thesis the sheep has been used as the experimental animal as it offers several advantages over the rodent. An important advantage from a practical point of view is that the larger size of the sheep ovary makes it more accessible for both *in vivo* and *in vitro* studies. This has enabled continuous sampling of ovarian venous effluent to be carried out *in vivo*, both *in situ* (Domanski, Skrzeczkowski, Stupnicka, Fitko and Dobrowolski, 1967; Moore, Barrett, Brown, Schindler, Smith and Smyth, 1969) and from autotransplanted ovaries

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(McCracken, Uno, Goding, Ichikawa and Baird, 1969). Its large size has also facilitated the development of techniques for isolating and culturing ovarian follicles (Moor, 1973) and follicular tissues (Moor, 1977). Follicles isolated in this way and maintained in organ culture continue to actively produce steroid hormones and to respond to gonadotrophins (Moor, Hay, McIntosh and Caldwell, 1973; Seamark, Moor and McIntosh, 1974; Moor, 1977). The similar size of sheep ovaries and ovarian follicles to those of other animals of agricultural importance and to the human, means that techniques developed for examining the sheep may be readily applied to studies of ovarian function in these other species. For these reasons, together with the ready availability of ovaries from a local abbatoirs, the isolated sheep follicle was used in the following studies.

The aim of the experiments described here was to characterise the changes in responsiveness of the sheep ovarian follicle to gonadotrophic stimulation. In addition, a preliminary investigation was made of the responsiveness of theca and granulosa cells of follicles from post partum and cycling cows.

This introduction describes some of the more recent findings related to changes in ovarian responsiveness that occur during developmental and cyclical maturation of ovarian tissue.

1.2 FOLLICULAR DEVELOPMENT

The commencement of growth of follicles from the primordial pool in the ovary occurs within a few days of birth in most species, although in primates it may begin before birth. Follicular growth begins when the single layer of flattened epithelial cells surrounding the oocyte of the primary follicle become cuboidal in form and proliferate mitotically

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to form the granulosa. At around the time the granulosa reaches four cell layers in thickness (Fig. 1, Type 5a - 5b) it becomes encapsulated by a sheath of tissue derived from the ovarian stroma. This sheath develops to become the theca. Fluid subsequently accumulates in the spaces between the granulosa cells and an antral follicle is formed (Fig. 1, Type 6 - 8).

Although the stimulus which initiates the selection of follicles from the pool of non-proliferating primary follicles is unresolved, it may be of intra-ovarian origin as follicle growth, at least in the mouse and rat, continues in the absence of gonadotrophins (Eshkol, 1970; Peters, Byskov, Himelstein-Braw and Faber, 1975; Nakano, Mizuno, Katayama and Tojo, 1975; Edwards et al. 1977). However, by the time the granulosa reaches about four cell layers in thickness FSH receptors can be demonstrated in the granulosa cells of the rat ovary and continued growth in the rat (for a review see Richards and Midgley, 1976) and mouse (Ryle, 1972; Peters et al. 1973) is dependent on the presence of gonadotrophins. Follicle growth in the neonatal mouse, prepubertal child (Peters et al. 1975) and in the neonatal ewe (Tassell, Chamley and Kennedy, 1978) continues into the antral stages but ovulation does not occur until puberty, or about 30 - 40 weeks of age in the ewe.

The number of follicles which begin to grow at any one time may be influenced by an inhibitory factor present in the follicular fluid of degenerating follicles (for a review see Peters, Byskov and Faber, 1973). As the neonatal mouse develops the increased number of growing follicles in the ovaries appears to reduce the number of follicles which leave the non-proliferating pool (Peters et al. 1973). A similar influence may exist in the sheep as the number of follicles beginning to grow falls at

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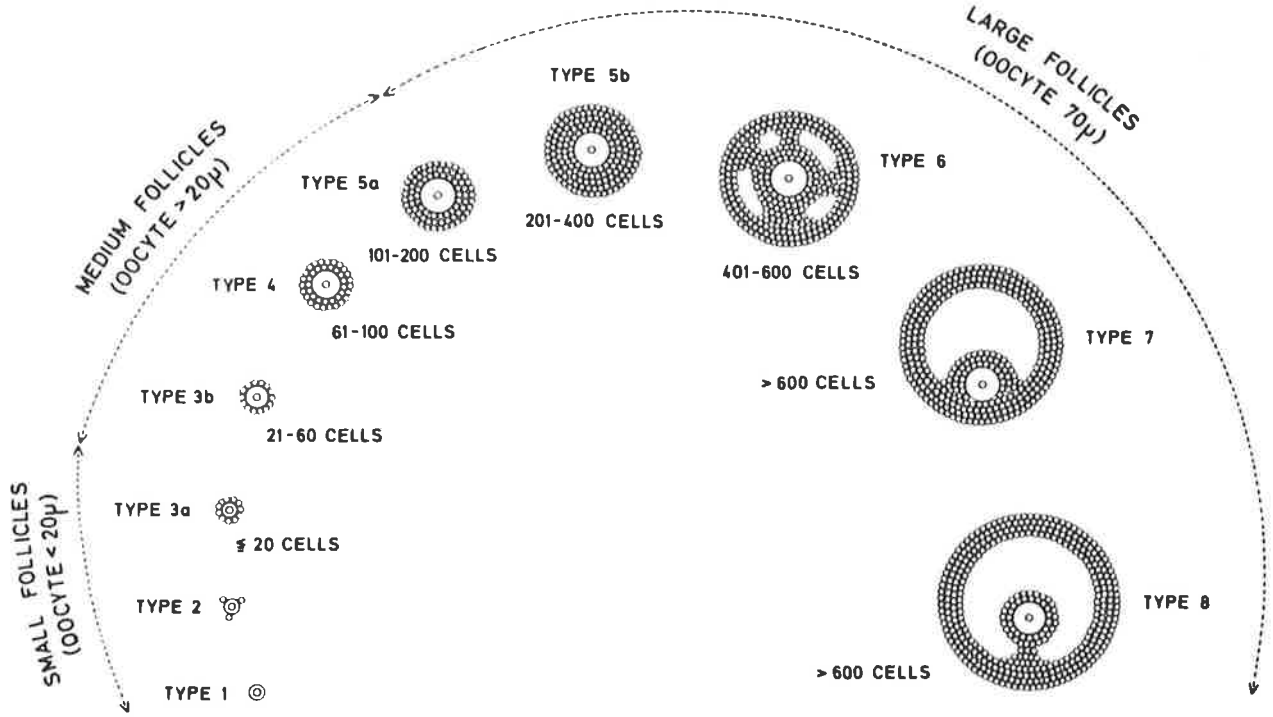

FIG. 1. Classification of follicles

From Pedersen and Peters, (1971).

about four weeks of age (Tassell et al. 1978). In addition, it has been proposed that in the mouse the number of follicles beginning to grow is influenced by the size of the primordial pool of follicles. (PETERS ET AL. 1973) This pool is reduced with advancing age and consequently the number beginning to develop also falls. However, this does not seem to be an important mechanism in the sheep, at least in the early stages of development, as the pool of primordial follicles does not decline significantly between birth and twenty weeks of age (Worthington and Kennedy, 1976).

The initiation of follicular growth occurs continuously throughout life. Although earlier studies in the sheep had suggested that follicular development was a cyclic phenomenon (Smeaton and Robertson, 1971; Brand and de Jong, 1973), there is now compelling evidence from the sheep (Turnbull et al. 1977), rat (Richards and Midgley, 1976) and mouse (Peters et al. 1975) that supports the proposal that follicles continually emerge from the pool of non-proliferating follicles, to begin growth regardless of the age of the animal, its reproductive state or the final fate of the follicle.

The appearance of gonadotrophin responsiveness in a follicle is a crucial step in its development, and the timing determines its ultimate fate. We now know that considerable variation in the binding of gonadotrophins occurs during development and maturation of the follicle (for a review see Channing and Kammerman, 1974). This variation relates to the stage of differentiation of the theca and granulosa, which is critical if a follicle is to respond appropriately to gonadotrophic stimulation. Follicles with the appropriate complement of gonadotrophin receptors and the biochemical machinery to enable them to respond to gonadotrophin binding are able to ovulate, while those that cannot respond

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are destined to become atretic (Richards and Midgley, 1976). The ovary of the postnatal rat (1 - 6 days old) does not respond to gonadotrophic stimulation although it does contain a prostaglandin E_2 (PGE_2)-responsive adenylate cyclase (Kolena, 1976). In this species it is not until the second week of life that the theca layer forms (Goldenberg, Reiter and Ross, 1973; Lunenfeld, Kraiem and Eshkol, 1975) and the ovaries acquire the ability to respond both to pregnant mare serum gonadotrophin [PMSG, a hormone with both LH and FSH-like activity, which causes ovarian weight gain (Goldenberg et al. 1973)] and to LH, which stimulates cyclic AMP production and increases the release of oestradiol-17β (for a review see Lindner et al. 1977). An illuminating example of the differing capacity of follicles of different sizes to respond to LH in the ovary of the adult cycling ewe was recently reported by Turnbull et al. (1977). Following the ovulatory LH surge in the ewe, or after treatment with PMSG followed 24 h later by hCG, the majority of antral follicles over 3.5 mm in diameter showed signs of luteinization, indicating that the granulosa in these follicles is responsive to LH stimulation. In contrast, smaller antral follicles largely appeared to be undergoing atresia.

The development of LH responsiveness by the granulosa is dependent on an FSH stimulated proliferation and maturation of the granulosa. In the rat, progressive follicular maturation results from an oestrogen stimulated binding of FSH and is associated with the appearance of LH receptors in the granulosa (Goldenberg et al. 1973). The number of LH (hCG) receptors in the granulosa is increased in immature or prepubertal (25 days of age) female, hypophysectomized rats by pre-treatment with oestradiol and FSH (Richards and Midgley, 1976; Rao, Richards, Midgley and Reichert, 1977) or by LH and FSH (Ireland and Richards, 1978).

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Further evidence of the need for co-operation between FSH and other ovarian influences was found by Nimrod, Tsafriri and Lindner, (1977) who demonstrated increased binding of ^{125}I-hCG to granulosa cells following exposure of rat ovarian fragments to FSH in organ culture. Incubation of granulosa cells alone with FSH did not increase the binding of ^{125}I-hCG. The concentration of LH receptors in the granulosa also increased as pig follicles increase in size (Channing and Kammerman, 1974; Kammerman and Ross, 1975; Nakano, Akahori, Katayama and Tojo, 1977), and is associated with an increase in the activity of an LH responsive adenylate cyclase (Lee, 1976). The formation of LH receptors in the granulosa under the influence of FSH has recently been shown to be enhanced in rats if the animals are pretreated with hCG (Ireland and Richards, 1978). While this effect does not appear to be exerted through a stimulation of oestradiol or testosterone levels, it is likely that this synergistic action of LH is mediated by a product of the theca layer as, at least in the rat, the theca contains LH receptors before the granulosa does (Presl, Pospisil, Figaravo and Wagner, 1972).

It is increasingly clear that successful follicular development depends on an intimate relationship between the theca and granulosa cells of the follicle. Both cell populations respond to circulating gonadotrophins at different times, depending on the stage of development, and as a result of that stimulation, may supply or utilize material from their adjoining cellular neighbour. The theca is the sole source of androgens in the ovaries of the hamster (Makris and Ryan, 1975), sheep (Seamark, Moor and McIntosh, 1974; Moor, 1977), and rat (Fortune and Armstrong, 1977). In the rat, incubation of isolated theca with highly purified LH (but not FSH) stimulates androgen production (Fortune and

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Armstrong, 1977). Furthermore, the addition of testosterone increases progesterone production of granulosa cells from immature ovaries of hypophysectomized, diethylstilbestrol-treated rats which are unresponsive to LH, as well as from pre-ovulatory follicles (Nimrod and Lindner, 1976; Luckey, Schrieber, Hillier, Schulman and Ross, 1977; Hillier, Knazek and Ross, 1977). Nimrod and Lindner (1976) have also shown a significant synergistic effect of testosterone or androstenedione on the steroidogenic action of FSH on granulosa cells in vitro. The added androgen does not appear to be merely providing substrate for aromatase activity (the enzyme system which converts androgens to oestrogens) because the addition of oestradiol or oestrone was without effect on progesterone synthesis. In contrast to these synergistic actions, androgens also inhibit oestrogen induced follicular growth and are implicated in the development of atresia (Louvet, Harman and Ross, 1975; Lunenfeld et al. 1975). In addition to the apparent regulatory role of androgens on granulosa cell steroidogenesis, androgens are the substrates for oestrogen synthesis by the follicle.

The site of oestrogen synthesis in the follicle is still a source of some controversy. In the hamster (Makris and Ryan, 1977) and possibly in follicles from hypophysectomized, immature rats, (Dorrington, Moon and Armstrong, 1975) the granulosa is the major site of aromatase activity. Isolated theca or granulosa prepared from sheep follicles secretes very little oestrogen compared to the intact follicle or follicle wall, indicating that close co-operation between the theca and granulosa is required for oestrogen synthesis (Moor, 1977). Evidence for an extracellular movement of androgen substrate from the theca to the granulosa has also been demonstrated indirectly in vivo as ovarian oestrogen secretion is

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reduced by infusion of testosterone antiserum (Baird, 1977). In contrast, the removal of theca and granulosa cells of the largest follicle of the monkey ovary *in vivo*, reduces ovarian oestrogen secretion while removal of granulosa cells alone does not, suggesting that in this species the theca is a major source of oestrogen (Channing and Coudert, 1976).

Oestrogen and FSH act synergistically to stimulate follicular growth. FSH stimulates the secretion of oestradiol-17β from hypophysectomized rat ovaries (Moon, Dorrington and Armstrong, 1975) and granulosa cells when incubated in the presence of androgen (Dorrington *et al.* 1975; for a review see Armstrong and Dorrington, 1977). A synthetic oestrogen, diethylstilbestrol, can stimulate the proliferation of granulosa cells of pre-antral rat follicles (Harman *et al.* 1975). Similarly, treatment of neonatal rats with PMSG is thought to increase ovarian weight gain by stimulating oestradiol production (Goldenberg *et al.* 1973). Treatment of female rats in the immediate post-natal period with specific anti-oestrogen serum causes a significant reduction in ovarian weight gain which is observable as early as the first post-natal week (Reiter, Goldenberg, Vaitukaitis and Ross, 1972). HCG inhibits ovarian weight gain and increases the number of atretic follicles in hypophysectomized rats, possibly as a result of increased androgen production (Louvet *et al.* 1975) or reduced oestrogen levels (Harman *et al.* 1975). Measurement of the steroidogenic activity of normal and atretic sheep follicles supports the view that a fall in oestrogen production is associated with atresia (Moor, Hay, Dott and Cran, 1978).

The majority of follicles which begin development never ovulate but become atretic and regress. Although follicles may become atretic at any stage of development, atresia is less common in growing pre-antral

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follicles than in antral follicles (Richards and Midgley, 1976; Byskov, 1977; for a review see Hay and Moor, 1978). Little is known about the physiology of atresia, largely because at the present time there is no satisfactory method of recognizing the incipient stages. The mechanisms responsible for initiating atresia are not known although it may result from increases in circulating gonadotrophins and consequent changes in ovarian steroid levels (Harman, Louvet and Ross, 1975; Hay and Moor, 1978). Studies in the sheep have suggested that changes in the thecal microcirculation which accompany atresia may have a role in its initiation, although it is difficult to determine whether these changes result in, or are a consequence of, atresia (Hay, Cran and Moor, 1976).

1.3 MECHANISM OF ACTION OF GONADOTROPHINS

The initial sites of action of polypeptide hormones such as LH and FSH are the plasma membranes of the target cells. The earliest consequence of the interaction between LH and its receptor is believed to be an activation of adenylate cyclase (Koch, Zor, Chobsieng, Lamprecht, Pomerantz and Lindner, 1974; see also reviews by Catt and Dufau, 1976 and by Kahn, 1976). Several workers have demonstrated that LH or hCG can stimulate cyclic AMP production by the ovaries or ovarian tissue of a number of species. These include the bovine corpus luteum (Marsh, Butcher, Savard and Sutherland, 1966), rabbit interstitial tissue (Dorrington and Baggett, 1969), mouse ovaries (Kuehl, 1970), rat ovaries (Mason, Schaffer and Toomey, 1973; Lamprecht, Zor, Tsafriri and Lindner, 1973), isolated ovarian follicles from the sheep (McIntosh and Moor, 1973), rat (Nilsson, Rosberg and Ahrén, 1974) and rabbit (Marsh, Mills and LeMaire, 1972), and pig granulosa cells (Kolena and Channing, 1972). Numerous other examples

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from a variety of ovarian preparations are to be found in the literature. FSH also increases intra-cellular cyclic AMP levels although differences in the rate of release of cyclic AMP from rat ovaries exposed to LH or FSH, as well as the observation that high concentrations of LH and FSH were additive, suggests that they have different sites of action in the ovary (Selstam, Rosberg, Liljekvist, Gronquist, Perklev and Ahrén, 1976). Changes in the sensitivity of rat ovarian homogenates to LH and FSH that are related to age also support the proposal that the two gonadotrophins have different sites of action (Fontaine, Salmon, Fontaine-Bertrand and Delerue-LeBelle, 1973).

Cyclic AMP, or its dibutyryl derivative, cause many of the changes in ovarian metabolism normally brought about by LH. Initiation of luteinization by cyclic AMP or dibutyryl cyclic AMP has been demonstrated in pig granulosa cells (Channing and Seymour, 1970), rat ovarian follicles (Ellsworth and Armstrong, 1973) and rabbit ovarian follicles (Miller and Keyes, 1974). In each case morphological luteinization was accompanied by the changes in steroid production normally begun by LH. Dibutyryl cyclic AMP is as effective as LH/FSH in stimulating progesterone and oestradiol production by isolated rabbit granulosa cells (Erickson and Ryan, 1975). It does not, however, induce luteinization or progesterone release in isolated sheep follicles (McIntosh and Moor, 1973) suggesting the involvement of other mechanisms. An earlier study by Le Maire, Mills, Ito and Marsh (1973) suggested that cyclic AMP may also inhibit luteinization, but the concentration of cyclic AMP used was far in excess of that normally found in tissues and may be the reason for this result.

The arrangement at each level of the hierachy of biochemical responses to gonadotrophic stimulation results in the ovary being very

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sensitive to changes in gonadotrophin levels, and secondly, and perhaps more important, it enables the ovary to considerably amplify a stimulatory signal. Firstly, there appears to be an excess of LH receptors in the ovary as occupation of only a fraction of the receptor sites is adequate to induce maximal production of cyclic AMP by the target tissue (Koch et al. 1974). This may be the result of receptors being arranged in 'domains' in which the receptors interact co-operatively with each other; binding of hormone to a receptor would result in activation of the whole domain of receptors (Biltonen, 1977). Secondly, the maximal production of cyclic AMP is far in excess of that required to maximally stimulate cyclic AMP-dependent protein kinase. However, the over production of cyclic AMP may only occur at particular times, for example, in response to a pre-ovulatory LH surge. During the rest of the cycle a more subtle influence is probably being exerted on the ovary. Marsh and Ling (1977) have recently demonstrated the existence of a close correlation between the activation of a cyclic AMP-dependent protein kinase and the stimulation of steroidogenesis in bovine corpus luteum slices. This is a particularly important observation as the activation occurred in response to a dose of LH which did not increase to a detectable level the concentration of cyclic AMP. This may explain why, in a recent study, low levels of hCG or glycosidase-treated derivatives of hCG apparently failed to increase the levels of cyclic AMP in Leydig cells but did increase toestosterone secretion (Bahl, 1977).

It is likely that the receptor component and the adenylate cyclase are not permanently linked but are free to move in the plasma membrane (for a review see Cuatrecasas, 1974). Studies on the transfer of β-adrenergic receptors from cell to cell by cell fusion techniques support

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this concept and show that the β-adrenergic receptor is independent of the catalytic adenylate cyclase component (Schulster, Orly and Schramm, 1978). Further, there is some evidence that the development of LH responsiveness is dependent on the formation of receptors which are then able to stimulate pre-existing adenylate cyclase. Kolena (1976) showed that the cyclic AMP levels in the newborn rat ovary can be increased by PGE_2 while LH is without affect. As the ovaries mature they acquire LH responsiveness, possibly as a result of the synthesis of LH receptors. The close correspondence between increased hCG binding and LH sensitive adenylate cyclase has been recently demonstrated by Salomon, Yanovsky, Mintz, Amir and Lindner (1977), in PMSG treated rats, and by Lee (1976) in pig granulosa.

While there is considerable evidence that cyclic AMP is an important mediator of hormone action it is becoming increasingly apparent that cell activation resulting from hormone binding to its receptor is the result of a complex series of interactions. For example, the activation of hepatic adenylate cyclase is dependent on the presence of divalent cations (Londos and Preston, 1977) and, at least in the ovary, adenylate cyclase is regulated by the relative concentrations of adenosine and guanosine triphosphate (Birnbaumer, Yang, Hunzicker-Dunn, Bockaert and Devan, 1976). In some cell systems cyclic AMP stimulates the release of bound calcium which then inhibits adenylate cyclase and stimulates phosphodiesterase activity (see the review by Rasmussen and Goodman, 1977). Further, there is increasing recognition that the original second messenger hypothesis (reviewed by Sutherland, Robison and Butcher, 1968), with cyclic AMP as sole intra-cellular mediator of hormone action, does not satisfactorily explain some metabolic responses observed in target

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tissues exposed to low concentrations of hormone (Schwyzer, 1974; see also the review by Catt and Dufau, 1977). However, in view of the close correlation between gonadotrophin binding, adenylate cyclase activation and the morphological and biochemical changes that result in the ovary, it is probable that a measure of adenylate cyclase, as assessed by changes in cyclic AMP levels, provides meaningful insight into the changes in gonadotrophin responsiveness of the ovary.

The specific aim of this thesis was to examine the effect of gonadotrophins on the production of cyclic AMP and steroids by isolated sheep follicles and follicular tissue, and by intact sheep ovaries in vivo at different stages of the oestrous cycle. In addition the possibility has been examined that post partum anoestrous in the cow is the result of a suppression of follicular responsiveness to gonadotrophins. The results of these studies are presented in Chapters 3 - 8.

Chapter 3 describes the changes in cyclic AMP in intact follicles induced by treatment with gonadotrophins. In Chapter 4 the effect is examined of hCG and FSH stimulation of cyclic AMP production by theca and granulosa cells isolated from follicles at two different stages of development. Chapter 5 describes experiments which assess the possibilities that gonadotrophins may regulate the responsiveness of follicular tissues to further gonadotrophic stimulation, and that this autoregulation may be responsible for the decline in androgen and oestrogen production reported to occur after the ovulatory LH surge. As a consequence of the latter study, in Chapter 6, a comparison is made of the responsiveness of theca and granulosa cells isolated from post partum and cycling cows.

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Chapter 7 describes the effects of hCG on the release of cyclic AMP and steroids from the sheep ovary in vivo at different stages of the oestrous cycle. Finally, in Chapter 8, an examination is made of the relationship between atresia and steroidogenesis in the isolated sheep follicle with the aim of developing an objective procedure for selecting non-atretic follicles for use in studies such as those which form the basis for the present thesis.

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CHAPTER 2

GENERAL METHODS

2.1 CYCLIC AMP ASSAY

Cyclic AMP was determined by saturation analysis using a binding protein contained in the supernatant from an homogenate of sheep adrenal cortex. This extract was prepared by the method of Brown, Albano, Ekins, Sgherzi and Tampion (1971), which was originally developed for extracting the binding protein from cattle adrenals. The sheep adrenal extracts gave excellent results over the range 0 - 20 pmol per assay tube when used at final dilutions of 1:120.

Each assay tube contained 50 µl of a solution of (^{3}H)adenosine-(G) 3' :5'-cyclic phosphate, ammonium salt [(^{3}H)-cyclic AMP; specific activity 22.1 Ci/mmol; New England Nuclear, Boston, Massachusetts, U.S.A.] containing approximately 5,000 cpm (0.31 pmol cyclic AMP), 100 µl binding protein solution and either a known amount of cyclic AMP standard (0 - 20 pmol; Sigma Chemical Co., St. Louis, Missouri, U.S.A.), or an appropriate portion of tissue extract made up to 200 µl with buffer[1] to give a final incubation volume of 350 µl.

The incubation tubes were allowed to equilibrate for 2 h at 4°, at the end of which free and bound cyclic AMP were separated by one of two methods. The method used for the experiments described in chapters 3, 4, 5, and 7 involved transferring 200 µl aliquots of the incubation mixture to small columns (1 by 3.5 cm) containing 0.5 g Sephadex G-25 fine, equilibrated with buffer. Elution was with buffer containing theophylline (Sigma). The void volume was discarded (0.5 ml) and the protein fraction eluted with 1 ml of buffer collected directly into scintillation vials. Scintillator fluid [10 ml Triton X-100:toluene-PPO-POPOP scintillator,

1. 50 mM Tris-HCI, pH 7.4, containing 8mM theophylline and 6 mM 2-mercapto-ethanol.

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1:2 (v/v)] was added and the radioactivity determined. This method proved satisfactory but was limited by the number of columns. In order to facilitate the assay of the large number of samples obtained from the experiments described in Chapter 6, charcoal separation was used (Brown et al. 1971). At the completion of the 2 h equilibration period 100 µl of a 0.5% suspension of charcoal (Norit A) in buffer containing 2% (w/v) of bovine serum albumin (BSA; fraction V, Calbiochem Aust. Pty. Ltd., Sydney, Australia) was added to each and the tubes briefly agitated. After centrifugation a 200 µl aliquot of the supernatant was taken for counting of radioactivity.

In all assays two unequal portions of extracts were measured to confirm that parallelism existed between the standard curve and the curve relating to the values obtained. The recovery of cyclic AMP added to either plasma or tissue homogenates was in excess of 80%. Testing with a wide range of nucleotides showed that the specificity of the sheep adrenal binding protein was very similar to that described for cattle adrenal tissue (Brown et al. 1971). The intra and inter-assay coefficients of variation were 12% (N = 36) and 15% (N = 40) respectively. The minimum amount of cyclic AMP that could be detected was 0.4 pmol.

Appropriate allowances were made for dilution and the results were expressed in pmol cyclic AMP per mg of follicular tissue or pmol cyclic AMP per mg of protein. Wet weights of follicular tissue were determined from mean follicular diameters using the relationship:

$$Y = 0.523\ [D^3 - (D - 0.209)^3]$$

where D = diameter of follicle in mm and Y = wet weight of tissue in mg. The relationship was derived by McIntosh and Moor and details are given by Weiss, Seamark, McIntosh and Moor (1976).

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2.2 STEROID ASSAYS

Progesterone, oestrogen, testosterone and androstenedione from culture medium and progesterone from ovarian vein plasma were all assayed by direct radioimmunoassay. The measurement of progesterone, oestrogen and testosterone in sheep peripheral plasma (Chapter 6) required extraction of 0.5 - 1 ml of plasma with 2 ml of redistilled diethyl ether. Standard curves were constructed by adding known amounts of steroid in ethanol to extraction tubes, evaporating to dryness at 37^{o} under nitrogen and then adding 0.5 - 1 ml of steroid free, charcoaled plasma to each tube. After a period of equilibration the standard tubes were extracted in a similar way to the unknowns. No adjustments were made for recovery because it was assumed that the losses in the standards were similar to those in the experimental samples. Each steroid was assayed by a radioimmunoassay procedure using polyethylene glycol 6000 for precipitation of the bound steroid (Janson, Amato, Weiss, Ralph and Seamark, 1978).

2.2.1 Progesterone assay: The progesterone antiserum was raised against progesterone conjugated through the 11 position to BSA and used at a dilution of 1:2000. The cross-reactivity of the progesterone antiserum was 5α-pregnanedione 5%, 20α-hydroxypregn-4-en-3-one 1%, 17α-hydroxyprogesterone 0.2%, testosterone 0.6%, oestradiol-17β, pregnanediol and pregnenolone < 0.1%. The limit of sensitivity was 0.05 ng.

2.2.2 Oestrogen assay: The oestrogen antiserum was raised against oestradiol-6-(O-carboxymethyl) oxime conjugated to BSA and used at a dilution of 1:16000. The cross-reactivity of the oestrogen antiserum with oestrone was 17%, oestriol 0.6%, testosterone 0.2%, and progesterone 0.1%. The

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limit of sensitivity was 25 pg.

2.2.3 Testosterone assay: The testosterone antiserum was raised against testosterone-3-(O-carboxymethyl) oxime conjugated to BSA and used at a dilution of 1:20000. The cross-reactivity of the testosterone antiserum with C_{18} and C_{21} steroids was less than 0.1% but for C_{19} steroids was androstenedione 1.3%, 5-dihydrotestosterone 31%, 4-androsten-3, 17-diol 30% and 4-androsten-17, 19-diol-3-one 3.5%. The term 'testosterone' is used in this thesis to include testosterone and the other cross-reactive androgens as suggested by Moor et al. (1975). The limit of sensitivity was 25 pg.

2.2.4 Androstenedione assay: The androstenedione antiserum was raised against androstenedione-3-(O-carboxymethyl) oxime conjugated to BSA and used at a dilution of 1:20000. The cross-reactivity of the androstenedione antiserum with C_{18} steroids was less than 0.1%, with C_{21} steroids was progesterone 0.5%, 17α-hydroxyprogesterone 0.2% and with C_{19} steroids was testosterone 0.4%, 5α-dihydrotestosterone < 0.8%. The limit of sensitivity was 25 pg.

The unlabelled steroids were obtained from Steraloids Inc., Pawling, New York, U.S.A. and radioactively labelled steroids from The Radiochemical Centre, Amersham, Bucks., U.K.

2.3 CULTURE OF FOLLICLES

Ovaries were removed from sheep of mixed breed (mainly Merino crossbreds) within 40 min of slaughter and transported to the laboratory in ice-chilled Dulbecco-phosphate buffered saline (CSL; Commonwealth Serum

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as judged by the appearance of the corpus luteum

Laboratories, Parkville, Victoria, Australia) containing 50 µg/ml kanamycin (Sigma). The following method is essentially the same as that reported by Moor et al. (1973).

Follicles between 1 and 3 mm and 4 and 6 mm in diameter were dissected from the ovaries of sheep between Days 4 and 14 of the oestrous cycle. The dissection was carried out under a stereoscopic microscope and in chilled Dulbecco-phosphate buffer containing 20% foetal calf serum (CSL). The preparation of up to 30 large follicles or 60 small follicles was usually completed within 3 h. The isolated follicles often* retained a small number of layers of stromal tissue which were not readily separated from the theca. The follicles were then separated into atretic or non-atretic groups using the morphological criteria described in Chapter 8 (this thesis) and with the exception of experiments described in Chapter 8, only non-atretic follicles were used.

Each intact dissected follicle was positioned on a platform of stainless steel mesh which was placed in a 1 cm well in a 3 cm sterile culture dish. Approximately 1 ml of culture medium, which consisted of 8 parts Medium 199 (CSL) and 2 parts foetal calf serum and containing 10 mg/l kanamycin & 56 mg/l insulin (bovine; CSL) was added to each culture dish. The amount of medium added was enough to reach the top of the platform. Vernier calipers were used to measure the diameter of the follicles after they had been positioned on the platforms.

The culture dishes were placed in a moist atmosphere consisting of 50% O_2, 5% CO_2, and 45% N_2 at 37° in McIntosh and Fildes' jar (Baird and Tatlock Ltd., Romford, Essex, U.K.) at a slight positive pressure.

The subsequent experimental procedure varied between experiments and is fully described in each chapter.

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2.4 GONADOTROPHIN PREPARATIONS

Luteinizing hormone, follicle stimulating hormone and prolactin were kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases. The preparations used were as follows:

Ovine, NIH-LH-S18	judged to contain less than 0.050 NIH-FSH-S1 units/mg[1].
Ovine, NIH-FSH-S10 NIH-FSH-S11	judged to contain less than 0.010 NIH-LH-S1 units/mg.
Ovine, NIH-P-S11	judged to contain less than 0.020 NIH-FSH-S1 units/mg and less than 0.0040 NIH-LH-S1 units/mg.

Human chorionic gonadotrophin was used in some experiments instead of LH as it is readily available, is relatively uncontaminated with other pituitary gonadotrophins and is reported to have a similar biological activity to LH. The preparation used was Pregnyl (Organon, Australia Pty. Ltd.) which is a purified, standardised preparation extracted from women during the first two months of pregnancy, when concentration of this hormone in the urine is maximal. Collection and purification at this time infers the absence of hormones with an FSH effect.[2] In addition, a vial of Pregnyl containing 500 i.u. of hCG was assayed for FSH using a h FSH radioimmunoassay kit supplied by The Radiochemical Centre, Amersham; there was no detectable FSH present.

1. Values obtained from information supplied by the NIAMDD.
2. Information supplied by Organon, Australia.

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2.5 STATISTICAL ANALYSIS

Where possible non-parametric statistics were used so as to avoid assumptions of normality and homoscedasticity in the data. Accordingly, sample medians are given together with 95% confidence limits on the population from which the sample was drawn using Nair's method and tables provided by Colquhoun (1971). Independent two-sample rank tests were done by Wilcoxon's method (also known as the Mann-Whitney U test) and k-sample tests by Kruskal-Wallis one-way analysis of variance. Related two-sample tests were done by the Wilcoxon signed ranks test and for k-related samples the Friedman two-way analysis of variance was used. All tests were done by computer, but Siegel (1956) and Colquhoun (1971) provide details for hand calculation. The latter author gives tables of critical ranges for determining where significant differences have occurred in analysis of variance by ranks. However, there is a lack of existing tables that allows for unequal group size. For this reason, a conservative procedure based on the Kruskal-Wallis test, that allows for unequal group size, was used where necessary. This method is described by Hollander and Wolfe (1973) and was also done by computer.

When interpreting the data presented in some of the figures, it must be remembered that the medians and 95% confidence limits are determined from the pooled group data and are therefore an estimate of both inter and intra experimental variation. Therefore, as the related or paired sample tests reduce the effect of inter-experimental variation, it is possible that treatments are significantly different even when the group confidence limits overlap the median of the other group.

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The extended Kruskal-Wallis test was used in the cases listed in the following tables:

3.4.1	3.4.6
3.4.2	3.4.8
3.4.4	3.4.9
3.4.5	6.4.2

Other tests are cited, where appropriate in the text.

Abbreviations used in the tables are as follows:

$\underline{m}$ = median

$\bar{R}$ = mean rank

95% c.l. = 95% confidence limits

N = number of replicates

N.S. = not significant

N.D. = not detectable.

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CHAPTER 3

CHANGES IN CYCLIC AMP LEVELS IN INTACT LARGE FOLLICLES AFTER TREATMENT WITH GONADOTROPHINS

3.1 INTRODUCTION

The role of cyclic AMP as a possible mediator of gonadotrophin action was first suggested by Marsh et al. (1966) as a result of studies with corpus luteum slices in which they found that the addition of dibutyryl cyclic AMP, a membrane permeable analogue of cyclic AMP, increased both ovarian steroidogenesis and glycogenolysis. For a review of the role of cyclic AMP in gonadal steroidogenesis see Marsh (1976). Since then LH has been shown to increase the cyclic AMP content of a number of ovarian preparations. There is now evidence that LH, through a receptor mediated activation of adenylate cyclase, activates a cyclic AMP-dependent protein kinase which in turn is thought to be responsible for the initiation of a variety of metabolic changes in the ovary. Some of these include stimulation of prostaglandin synthetase, cholesterol esterase and cholesterol transport, conversion of cholesterol to pregnenolone, increase in protein and RNA synthesis, lactic acid production and glucose oxidation (review, Channing and Tsafriri, 1976; Marsh, 1976). FSH also influences the ovary through a receptor mediated stimulation of adenylate cyclase.

Prostaglandins (PG) are also thought to have a role in mediating the effects of LH on the ovary as they are capable of similar actions, including initiation of steroidogenesis. However, failure of inhibitors of prostaglandin synthesis to abolish LH action and differences in the chronologic sequence of LH and prostaglandin action suggests that prostaglandins are not obligitory intermediaries of LH action as proposed

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by Kuehl (1974). Gonadotrophins do increase ovarian prostaglandin levels in rat Graafian follicles in vitro (Bauminger, Lieberman and Lindner, 1975) and in vivo where ovarian levels reach a peak on the morning of oestrus (Bauminger and Lindner, 1975). As a result it is proposed that ovarian prostaglandins are necessary for follicular rupture but do not appear to be involved in ovum maturation or luteinization of the granulosa cells (review, Goldberg and Ramwell, 1975).

The influence of nerves and catecholamines on ovarian function is unclear. There are numerous reports supporting and denying their importance in the ovary (review, Bahr, Kao and Nalbandov, 1974). However, the occurrence of smooth muscle cells (Okamura, Virutamasen, Wright and Wallach, 1972) and autonomic nerves in the human follicle wall indicates a possible role for nervous influence in ovulation (Owman, Sjoberg, Svensson and Walles, 1975). In addition, noradrenaline and adrenaline stimulate progesterone production by cow corpus luteum through a beta-adrenergic receptor suggesting the possibility of a nervous influence on steroidogenesis (Condon and Black, 1976).

3.2 AIM

This study was undertaken to examine the possible role of cyclic AMP production in the LH and FSH stimulation of the isolated preovulatory sheep follicle. Secondly, to determine the effect on follicular levels of cyclic AMP of several other hormones thought to influence the ovary.

3.3 METHODS

Ovaries were removed from sheep of mixed breed (mainly Merino cross-breds) within 40 min of slaughter and transported to the laboratory in ice-chilled Dulbecco phosphate-buffered saline containing kanamycin (50 µg/ml). Follicles between 4 and 6 mm in diameter (usually 2 per ovary)

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were dissected from ovaries of sheep between days 4 and 14 of the oestrus cycle and established in organ culture as described by Moor et al. (1973). After an initial 18 - 24 hour culture period in gonadotrophin free medium, the follicles were randomly allocated to the experimental groups.

The incubation of follicles was carried out under the same conditions as for the 18 hour preincubation except that unless otherwise indicated 8 mM theophylline was included in the incubation medium. Experimental incubations were begun by the addition of a small volume of medium containing an appropriate concentration of the hormone being examined. At the end of the culture period the medium was removed and frozen. The follicles were homogenized in an all glass homogenizer containing 1 ml of ice cold 50 mM - Tris HCl buffer, pH 7.4 and 8 mM theophylline and 6 mM 2-mercaptoethanol. Protein in the samples was precipitated by the addition of 3 vol of ethanol and the protein free supernatant then assayed for cyclic AMP or stored at -20^{o}C until assayed. A slightly modified version of the competitive protein binding assay of Brown et al. (1971) was used to estimate cyclic AMP values. (See Chapter 2, this thesis, for full description of method).

Results are expressed as pmol cyclic AMP/mg of follicular tissue. The wet weight of follicular tissue were determined from the mean follicular diameter using a relationship derived by McIntosh and Moor (Weiss et al. 1976).

Non-parametric statistics were used to ascertain the significance of differences between groups (see Chapter 2 on analysis of results). Results are gives as group medians with 95% confidence limits.

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3.4 RESULTS

Basal Cyclic AMP Levels: The median tissue content of cyclic AMP in 21 unstimulated follicles determined after 18 hours in culture was 0.3 (0.3 - 1.1) pmol/mg and the amount in the medium was equivalent to a release of 0.2 (0.1 - 0.6) pmol/mg).

3.4.1 Experiment 1: Aim: To establish a time course of cyclic AMP production following LH and FSH stimulation.

Results: After the addition of LH (NIH-LH-S18; 50 µg/ml) and FSH (NIH-FSH-S10; 50 µg/ml) there was a rapid rise in tissue cyclic AMP and a subsequent increase in the level of cyclic AMP in the medium (Fig. 3.4.1 and Tables 3.4.1 and 3.4.2). The tissue level of cyclic AMP was significantly higher than control level at 40 min in both LH and FSH treated groups. The concentration of cyclic AMP in the medium rose during the incubation with both hormones and was higher ($P < 0.05$) than the control after 40 min. There was no evidence that the tissue level of cyclic AMP differed between follicles treated with FSH or LH during the first 40 - 90 min (Table 3.4.3).

However, after 90 min the concentration of cyclic AMP was higher ($P < 0.05$) in the medium of follicles treated with LH compared to those treated with FSH (Table 3.4.3). Exposure of follicles to hCG (20 i.u./ml) resulted in an increase in the cyclic AMP level in tissue and medium similar to that seen after LH (Fig. 3.4.2 and Table 3.4.4), although hCG increased levels in the tissue faster than LH while LH appeared to increase levels in the medium faster than hCG. However, the lack of equivalent doses of LH and hCG makes interpretation difficult.

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Table 3.4.1: Time course of changes in tissue and medium levels of cyclic AMP after incubation with FSH (50 ug/ml).

Treat. (min)	Tissue m	95% conf. l.	$\bar{R}$	Medium m	95% conf. l.	$\bar{R}$	N
0	0.3	(0.2 - 0.4)	8.6	0.8	(0.6 - 1.4)	12.9	15
5	1.0	(0.7 - 1.7)	17.6	0.3	(0.1 - 1.0)	6.3	6
40	18.0	(11.3 - 53.2)	31.8	6.1	(3.1 - 7.3)	30.3	12
60	12.0	(1.4 - 16.9)	25.5	5.2	(2.5 - 7.9)	28.8	4
90	44.6	(14.7 - 70.7)	38.0	12.4	(6.9 - 16.6)	39.3	6

The following pairs of groups are significantly different at the P = 0.05 level (modified Kruskal-Wallis):

0 and 40 min	0 and 40 min
0 and 90 min	0 and 90 min
5 and 90 min	5 and 40 min
	5 and 90 min

Table 3.4.2: Time course of changes in tissue and medium levels of cyclic AMP after incubation with LH (50 ug/ml).

Treat. (min)	Tissue m	95% conf. l.	$\bar{R}$	Medium m	95% conf. l.	$\bar{R}$	N
0	0.3	(0.2 - 0.4)	8.5	0.8	(0.6 - 1.4)	13.5	15
5	1.5	(0.5 - 14.0)	19.4	0.4	(0.1 - 1.0)	6.8	5
15	4.6	(4.5 - 7.7)	21.0	1.1	(0.5 - 1.7)	15.8	4
40	25.6	(11.6 - 50.4)	38.7	6.6	(2.5 - 12.0)	31.4	9
60	16.5	(4.7 - 53.8)	34.4	14.9	(4.7 - 53.8)	37.1	7
90	35.1	(13.4 - 113)	44.7	20.8	(13.4 - 50.7)	42.6	7
120	23.3	(16.3 - 27.6)	37.0	30.6	(25.2 - 58.3)	47.3	4
180	34.9	(26.8 - 71.2)	45.5	58.0	(45.1 - 66.6)	52.5	4

The following pairs of groups are significantly different at the P = 0.05 level:

0 and 40 min	0 and 60 min	5 and 90 min
0 and 60 min	0 and 90 min	5 and 120 min
0 and 90 min	0 and 120 min	5 and 180 min
0 and 120 min	0 and 180 min	15 and 180 min
0 and 180 min	5 and 60 min	

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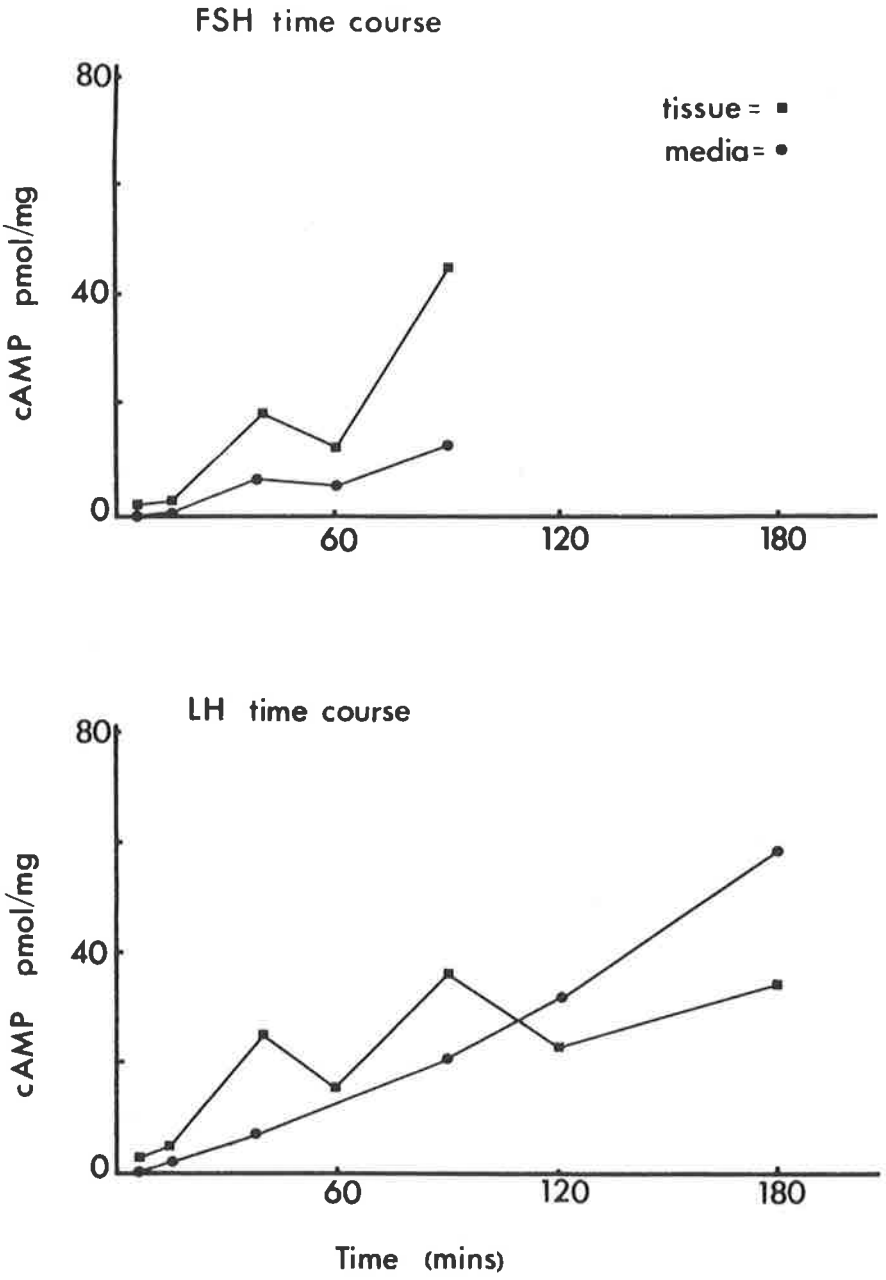

Figure 3.4.1 Time course of changes in tissue and medium levels of cyclic AMP after incubation of intact, large follicles with LH or FSH (50 ug/ml).

Values plotted are medians. See Tables 3.4.1 and 3.4.2 for details.

Table 3.4.3: Comparison of the effect of LH and FSH on the cyclic AMP levels in intact, large follicles.

Dose-response (40 min incubation)		
Dose (ug/ml)	Tissue	Medium
20	LH > FSH P - 0.04[1]	N.S.[2]
50	N.S.	N.S.
100	LH > FSH P = 0.004	LH > FSH P = 0.07
200	N.S.	N.S.
Time course (dose = 50 ug/ml)		
Time (min)	Tissue	Medium
5	N.S.	N.S.
40	N.S.	N.S.
60	N.S.	N.S.
90	N.S.	LH > FSH P = 0.05

1. Probability value (Mann-Whitney) arising from the comparison of LH and FSH treatment groups.

2. Not significant.

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Table 3.4.4: Time course of changes in tissue and medium levels of cyclic AMP following incubation of intact, large follicles with hCG (20 i.u./ml).

Treat. (min)	Tissue m	95% conf. l.	$\bar{R}$	Medium m	95% conf. l.	$\bar{R}$	N
0	0.3	(0.2 - 0.4)	8.0	0.8	(0.6 - 1.4)	13.0	15
5	11.7	(3.4 - 24.0)	23.7	1.2	(0.5 - 5.7)	22.0	11
40	29.3	(20.5 - 43.3)	38.5	3.7	(1.8 - 23.3)	34.4	13
60	31.1	(11.7 - 43.6)	39.2	4.8	(3.6 - 8.2)	36.7	10
90	44.9	(23.3 - 65.4)	47.2	11.2	(10.4 - 12.6)	48.0	5
180	55.7	(37.7 - 96.3)	52.5	34.2	(24.3 - 38.6)	55.8	5

The following pairs of groups are significantly different at the P = 0.05 level:

Tissue	Medium
0 and 40 min	0 and 40 min
0 and 60 min	0 and 60 min
0 and 90 min	0 and 90 min
0 and 180 min	0 and 180 min
5 and 180 min	5 and 180 min

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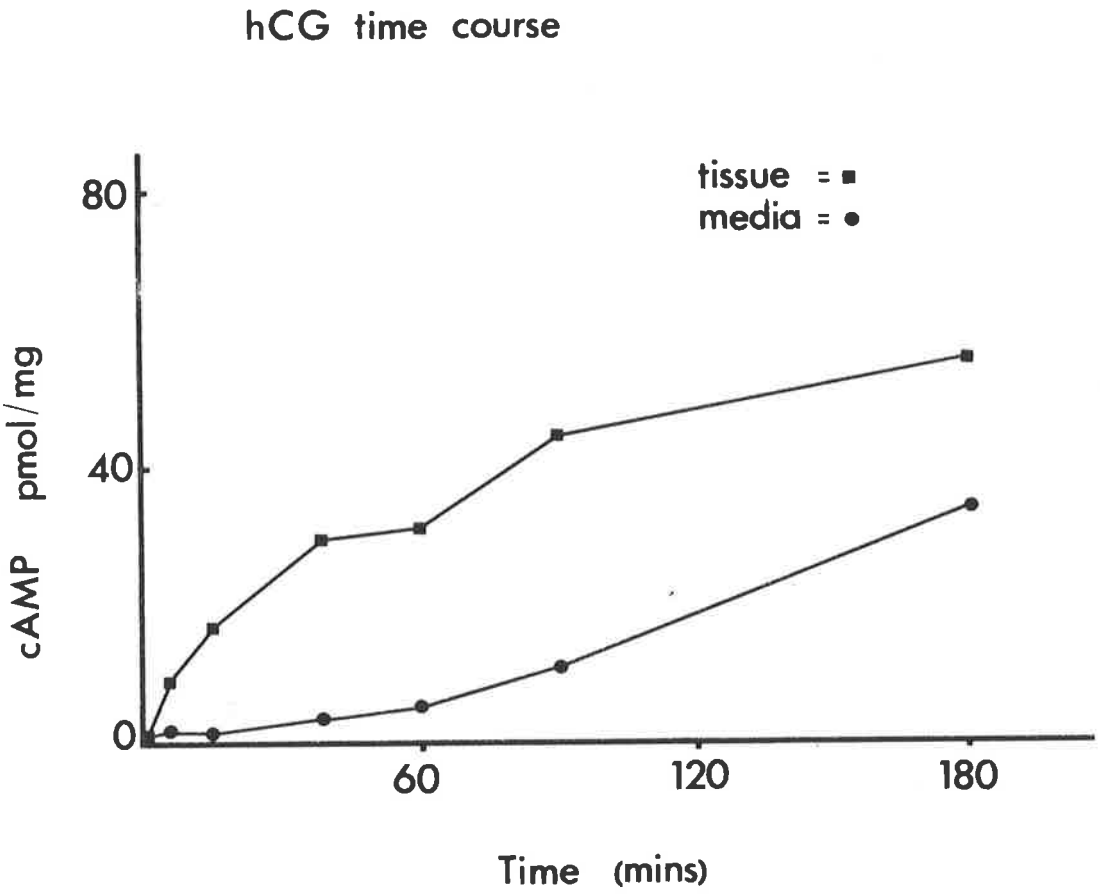

Figure 3.4.2 Time course of changes in tissue and medium levels of cyclic AMP following incubation of intact, large follicles with hCG (20 i.u./ml).

Values plotted are medians. See Table 3.4.4 for details.

3.4.2 Experiment 2: Aim: To determine a dose response relationship between different concentrations of LH or FSH and cyclic AMP levels.

Results: Intact follicles were incubated for 40 min in the presence of concentrations of LH or FSH varied from 0 - 200 µg/ml. The cyclic AMP level in the tissue and medium rose as concentrations of both hormones were increased. The addition of LH or FSH at concentrations of 50, 100 and 200 µg/ml increased the tissue level of cyclic AMP over the control. The tissue response was reflected in the medium; interestingly, the level in the medium was higher after incubation with 20 µg/ml of LH than after a similar dose of FSH. While the medium tissue cyclic AMP level and mean rank (Table 3.4.5 and 6) increased as the dose of LH and FSH was increased from 50 - 200 µg/ml the variability of the response was such that the differences did not reach significance ($P > 0.05$).

3.4.3 Experiment 3: Aim: To examine the effect of melatonin, serotonin, noradrenaline, prolactin and progtaglandin E_2 (PGE_2) on follicular cyclic AMP levels.

Results: Follicles were incubated for 40 min (except the PGE_2 treated group which was incubated for 20 min) in the presence of either melatonin (20 µg/ml; Sigma), serotonin (20 µg/ml; Sigma), noradrenaline (20 µg/ml; DL - arterenol hydrochloride: Sigma), prolactin (50 µg/ml; NIH-P-S11; 26.4 i.u./mg) and PGE_2 (20 µg/ml; U-12062: Upjohn Pty. Ltd., Parramatta, N.S.W., Australia). It was found that noradrenaline and PGE_2 altered cyclic AMP levels in both the tissue and medium

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Table 3.4.5: Effect of increasing doses of FSH on cyclic AMP levels in intact, large follicles and medium following a 40 min incubation.

Treat. (ug/ml)	Tissue m	95% conf. l.	$\bar{R}$	Medium m	95% conf. l.	$\bar{R}$	N
0	0.3	(0.2 - 0.4)	8.0	0.8	(0.6 - 1.4)	11.5	15
2	3.3	-	17.0	0.6	-	5.7	3
20	9.6	(7.3 - 15.2)	23.5	1.2	(1.0 - 1.3)	15.8	4
50	18.0	(11.3 - 53.7)	33.3	6.1	(3.1 - 7.3)	37.3	12
100	22.2	(11.1 - 37.0)	34.3	4.1	(2.4 - 5.1)	30.2	10
200	67.1	(16.7 - 92.1)	42.3	7.7	(5.7 - 11.3)	43.5	4

The following pairs of groups are significantly different at the P = 0.05 level:

0 and 50 ug/ml	0 and 50 ug/ml	2 and 50 ug/ml
0 and 100 ug/ml	0 and 100 ug/ml	2 and 200 ug/ml
0 and 200 ug/ml	0 and 200 ug/ml	

Table 3.4.6: Effect of increasing doses of LH on cyclic AMP levels in intact follicles and medium following a 40 min incubation.

Treat. (ug/ml)	Tissue m	95% conf. l.	$\bar{R}$	Medium m	95% conf. l.	$\bar{R}$	N
0	0.3	(0.2 - 0.4)	8.0	0.8	(0.6 - 1.4)	8.5	15
2	16.6	-	19.7	4.0	-	21.3	3
20	19.3	(14.3 - 50.2)	26.5	8.1	(7.7 - 11.7)	33.8	4
50	25.6	(11.6 - 50.4)	26.1	6.6	(2.8 - 12.0)	29.4	9
100	47.3	(23.1 - 97.4)	35.7	5.3	(1.4 - 10.3)	29.7	9
200	79.4	(42.8 - 156)	40.0	12.3	(5.3 - 17.8)	37.0	6

The following pairs of groups are significantly different at the P = 0.05 level:

0 and 50 ug/ml	0 and 20 ug/ml
0 and 100 ug/ml	0 and 50 ug/ml
0 and 200 ug/ml	0 and 100 ug/ml
	0 and 200 ug/ml

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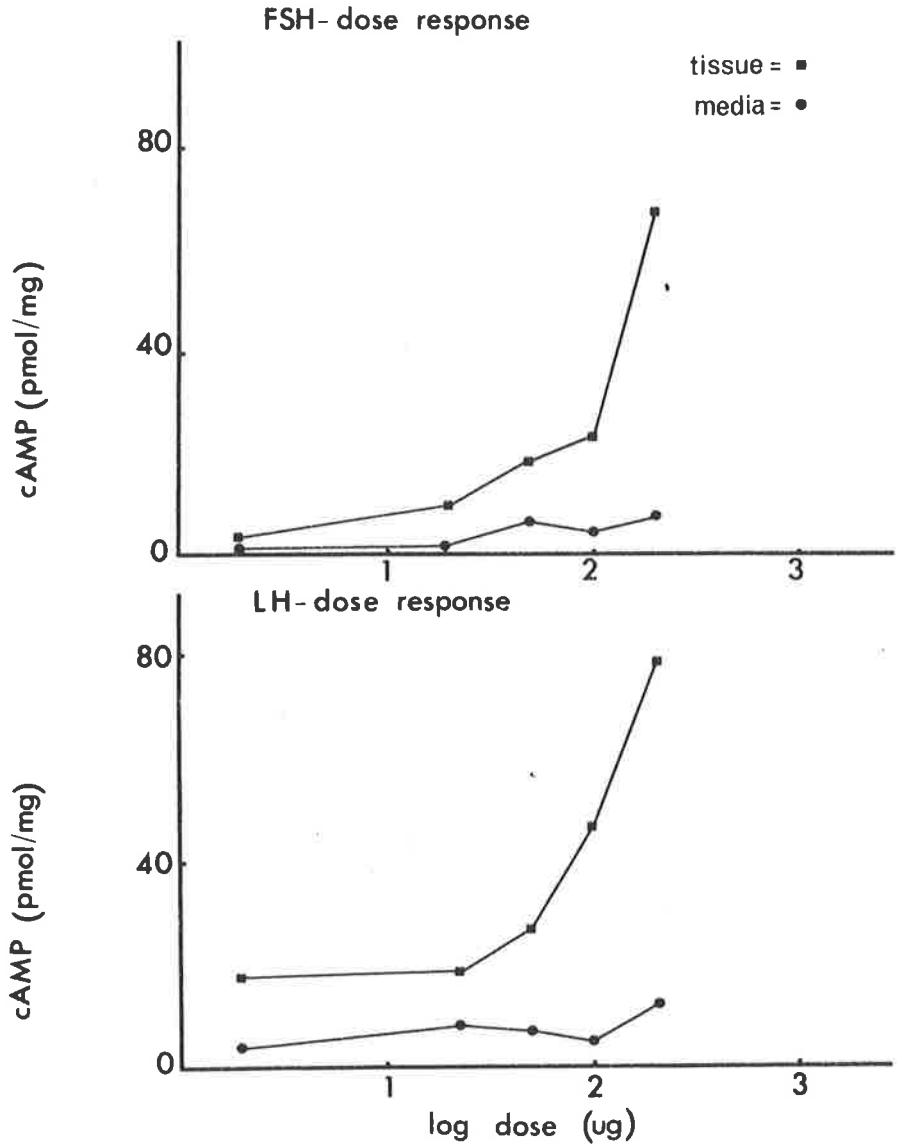

Figure 3.4.3 Dose-response relationship between LH and FSH and cyclic AMP levels in intact, large follicles and incubation medium after 40 min incubation.

Values plotted are medians. See Tables 3.4.5 and 3.4.6 for details.

(Table 3.4.7) whereas prolactin increased the cyclic AMP in the medium alone. Serotonin and melatonin had no discernible effect.

Table 3.4.7: Formation of cyclic AMP [median (95% confidence limits)] by large follicles in culture for 40 min in response to hormones added to the medium.

Hormone	Dose (per ml of medium)	No. of follicles	cyclic AMP pmol mg	
			Tissue	Medium
none (control)	-	15	0.3 (0.2 - 0.4)	0.8 (0.5 - 1.4)[1]
prolactin	50 µg	6	0.6 (0.6 - 1.7)[1]	1.8 (1.2 - 5.1)[1]
prostaglandin E_2[2]	20 µg	5	4.9 (1.6 - 6.2)[1]	5.8 (3.0 - 8.6)[1]
noradrenaline	20 µg	6	2.8 (1.0 - 5.1)	4.1 (1.6 - 6.6)[1]
serotonin	20 µg	6	0.4 (0.2 - 0.5)	1.6 (0.9 - 2.4)
melatonin	20 µg	6	0.3 (0.1 - 0.5)	1.2 (0.5 - 1.9)

1. Treatments significantly different to control, $P < 0.05$ by Mann-Whitney U test.
2. Incubation time = 20 min.

3.4.4 Experiment 4: Aim: To establish a dose response and time course relationship between noradrenaline and follicular cyclic AMP levels.

Results: The tissue content of cyclic AMP was increased after incubation of follicles with concentrations of noradrenaline of 1, 10 and 100 µg/ml. In contrast to the effect of LH, FSH and hCG described earlier, the rise in tissue levels was not accompanied by a release of cyclic AMP into the medium (Fig. 3.4.4, Table 3.4.8).

When the follicles were incubated with noradrenaline (20 µg/ml) for varying times the cyclic AMP levels reached a maximum after 10 min (Fig. 3.4.5, Table 3.4.9). This level was similar after 20, 40 and 90 min incubation although there was an inexplicable fall after 60 min. Again

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in contrast to the effect of gonadotrophins, noradrenaline did not increase the level of cyclic AMP in the medium; in fact after 10 min the concentration of cyclic AMP in the medium from treated follicles was significantly lower ($P < 0.05$) than in the medium from control follicles.

" Note on 'control' follicles in this chapter.

The median tissue content of cyclic AMP and the median concentration of cyclic AMP in the medium after 18 h in culture is given on pg 26 (3.4) and was 0.3 and 0.2 pmol/mg respectively. In the experiments reported subsequently tissue and medium cyclic AMP levels determined in both follicles that were not incubated and in follicles that were incubated for up to 180 min. The values obtained were consistenly low and close to the limits of sensitivity of the assay. There was no significant difference between the tissue content of of non-incubated follicles (ie. '0' time) and of follicles incubated for up to 180 min. without hormone treatment. For this reason, and in order to reduce the variation within the control group, these values were pooled to give the 'control' value used in the statistical analyses presented in Tables 3.4.1, 3.4.2, 3.4.4, 3.4.5, 3.4.6, 3.4.7, 3.4.8 and 3.4.9."

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Table 3.4.8: Effect of increasing doses of noradrenaline on cyclic AMP levels in intact, large follicles and medium following a 20 min incubation.

Treat. (ug/ml)	Tissue m	95% conf. l.	$\bar{R}$	N
0	0.3	(0.2 - 0.4)	8.0	15
0.01	4.4	(3.3 - 6.4)	20.8	5
0.1	5.3	(2.8 - 6.5)	21.8	5
1.0	7.4	(5.0 - 8.9)	28.0	5
10.0	10.3	(9.5 - 30.6)	35.6	5
100.0	13.9	(5.3 - 17.7)	33.6	5

The following pairs of groups are significantly different at the P = 0.05 level:

0 and 1.0 ug/ml

0 and 10.0 ug/ml

0 and 100.0 ug/ml

The concentration of cyclic AMP in the medium did not differ from control at any of the doses of noradrenaline tested.

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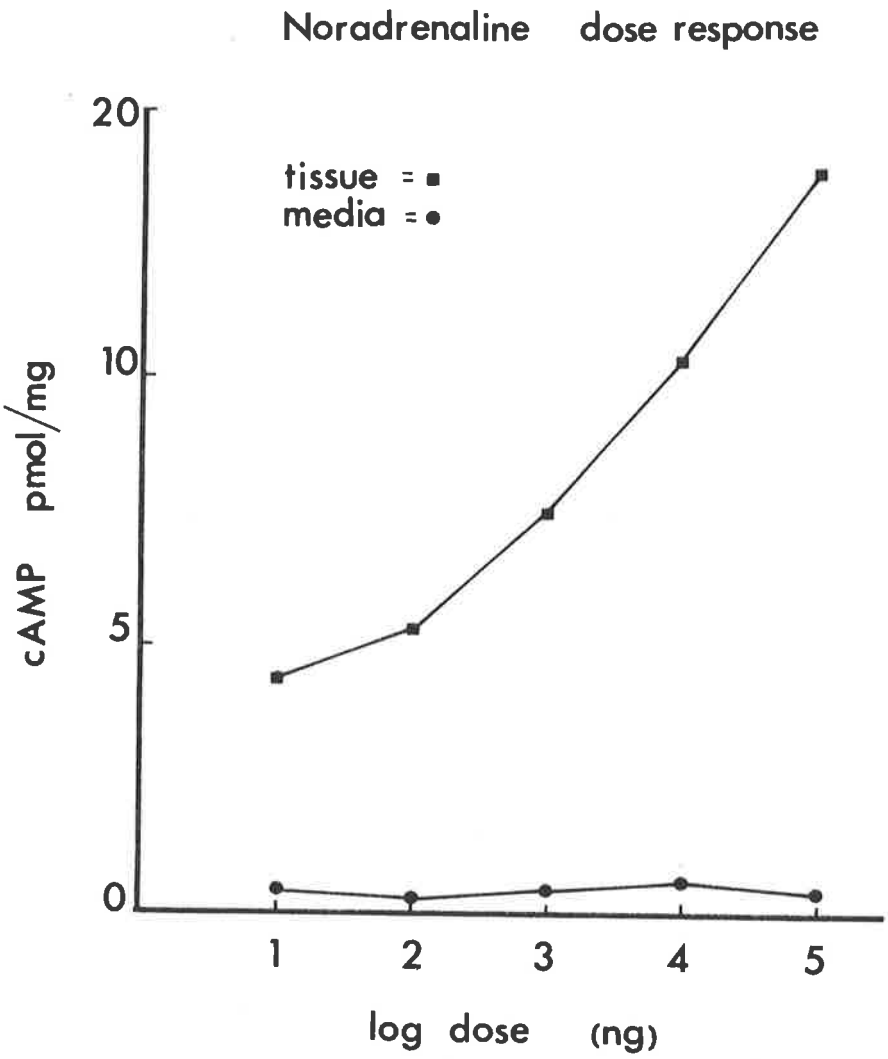

Figure 3.4.4 Dose-response relationship between noradrenaline and cyclic AMP levels in intact, large follicles and incubation medium after 20 min incubation.

Values plotted are medians. See TAble 3.4.8 for further details.

Table 3.4.9: Time course of changes in tissue and medium levels if cyclic AMP following incubation of intact, large follicles with noradrenaline (20 ug/ml).

Treat. (min)	Tissue m	Tissue 95% conf. l.	Tissue $\bar{R}$	Medium m	Medium 95% conf. l.	Medium $\bar{R}$	N
0	0.3	(0.2 - 0.4)	8.1	0.8	(0.6 - 1.4)	53.1	15
5	4.0	(2.1 - 4.8)	31.2	-			5
10	9.4	(3.2 - 26.0)	46.5	0.6	(0 - 0.9)	24.6	11
20	8.9	(4.2 - 15.1)	48.4	0.6	(0.2 - 0.9)	36.3	14
40	8.1	(2.4 - 13.6)	45.2	0.5	(0.2 - 0.6)	34.9	11
60	4.1	(2.7 - 6.7)	32.3	0.5	(0.2 - 1.5)	41.8	6
90	7.8	(3.6 - 13.7)	45.5	0.4	(0.3 - 1.5)	41.0	10

The following pairs of groups are significantly different at the P = 0.05 level:

0 and 10 min	0 and 10 min*
0 and 20 min	
0 and 40 min	
0 and 90 min	

* The level at 10 min was lower than control.

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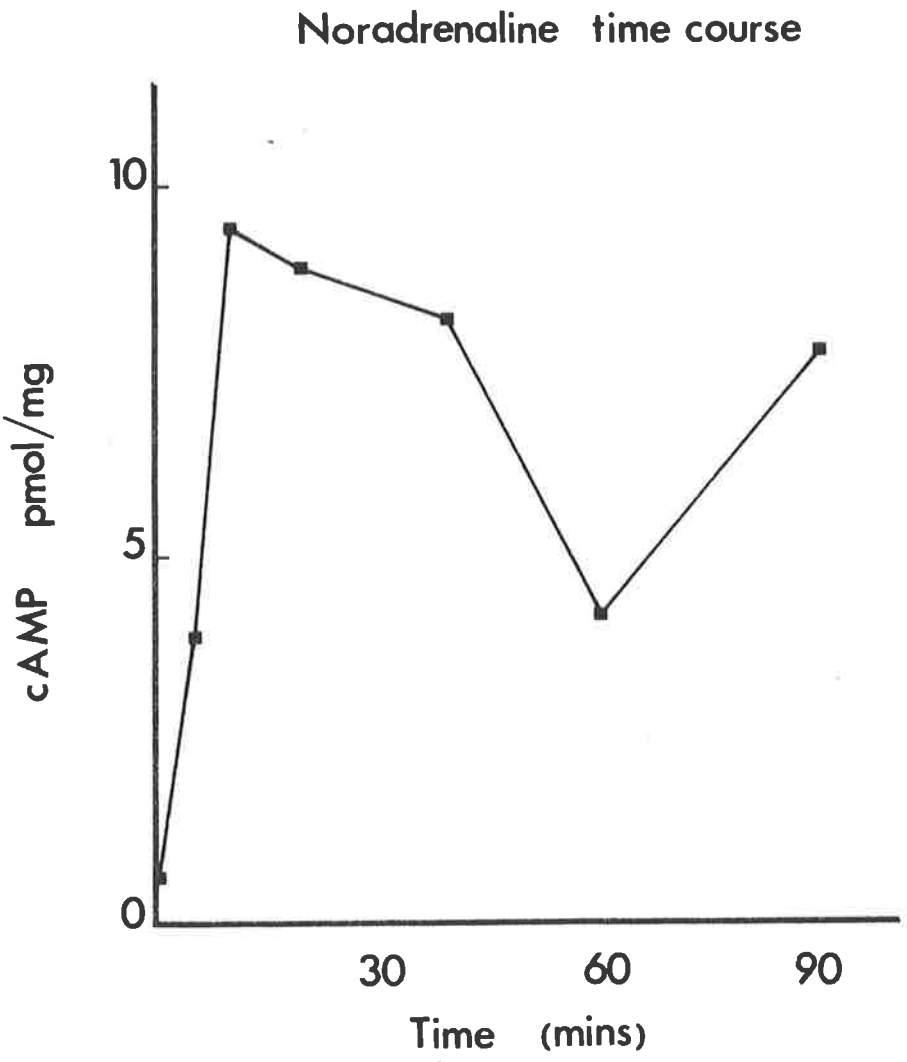

Figure 3.4.5 Time course of changes in tissue cyclic AMP levels after incubation of intact, large follicles with noradrenaline (20 ug/ml).

Values plotted are medians. See Table 3.4.9 for details.

3.5 DISCUSSION

These results show that there is a increase in the synthesis of cyclic AMP by sheep ovarian follicles after both LH and FSH stimulation, and that, this increase in tissue levels is accompanied by a significant release of cyclic AMP into the medium. The increase in cyclic AMP levels precedes the changes in follicular steroidogenesis (McIntosh and Moor, 1973), which are thought to be initiated by an activation of a cyclic AMP dependent protein kinase system. Some of the changes in steroidogenic activity occur very rapidly; for example, PMSG significantly increases oestradiol production from large sheep follicles within 5 minutes (Moor et al. 1973). An increase in progesterone production by sheep follicles, on the other hand takes more than 24 hours, by which time cyclic AMP levels will have declined to prestimulation levels (data not shown). Marsh (1976) has suggested five different pathways through which a cyclic AMP-dependent protein kinase may influence steroidogenesis; namely by increasing co-factor availability, increasing substrate availability, facilitating cholesterol transport, increasing side chain cleavage or improving the efflux of pregnenolone from mitochondria. The length of time required for these metabolic alterations to the steroid secreting cell will vary, but will clearly be significant if RNA and protein synthesis is required. The induction of the aromatase system in the immature rat ovary requires about 12 hours of gonadotrophic stimulation and is prevented by cycloheximide, an inhibitor of protein synthesis (Sashida and Johnson, 1976). However, the authors conclude that cyclic AMP may not be involved in the induction of the aromatase system as a single dose of FSH + LH produced the same increase in cyclic AMP as PMSG but the former did not increase oestradiol levels. Their

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conclusion highlights the problem of interpreting results from studies on whole ovaries where it is not possible to determine the source of the ovarian cyclic AMP or the site(s) of action of the two gonadotrophin preparations used. Differences in the concentration of cyclic AMP in the tissue and medium of incubated immature rat ovaries after LH and FSH treatment suggests that the two hormones have different sites of action. (Selstam et al. 1976).

Our observation that the level of cyclic AMP in the medium was greater after 90 min incubation with LH than FSH supports this proposal although the differences were not as striking as those in the previous study.

The observation that significant quantities of cyclic AMP were released into the medium raises several questions about its extracellular role. Release of cyclic AMP into the extracellular compartment has been observed in a wide variety of tissues including erythrocytes (Davoren and Sutherland, 1963), rat liver (Broadus, Kaminsky, Northcutt, Hardman, Sutherland and Liddle, 1970), adrenal (Carchman, Jaanus and Rubin, 1971; Peytremann, Nicholson, Hardman and Liddle, 1973), testis (Dufau, Watanabe and Catt, 1973) and prepubertal rat ovaries stimulated with gonadotrophin or PGE_2 (Nilsson, Rosberg and Ahrén, 1974). It is likely that the release of cyclic AMP is part of the mechanism for rapidly reducing intracellular cyclic AMP levels. Cyclic AMP produced by prepubertal rat ovaries in vitro is degraded by an extracellularly active, membrane bound phosphodiesterase (Rosberg, Selstam and Ahrén, 1974). This may also be the case in the sheep as the inclusion of theophylline, an inhibitor of phosphodiesterase, potentiated cyclic AMP levels, particularly in the medium (Weiss et al. 1976). However, the possibility of an extra-

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cellular role for cyclic AMP cannot be discounted.

The role of nervous innervation of the ovary in ovulation is not clear. Numerous studies have produced results both in support of, or against, a role for nervous activity in ovulation (review - Bahr et al. 1974). Both adrenaline and noradrenaline, through a β-adrenergic receptor stimulate progesterone synthesis by bovine corpus luteum (Condon and Black, 1976). They also cause contraction of isolated sheep follicle wall but in contrast to the effect on steroidogenesis this is mediated by an α-adrenergic mechanism (O'Shea and Phillips, 1974). The stimulatory effect of noradrenaline on cyclic AMP formation is indicative of β-adrenergic stimulation and suggests the possible involvement of catecholamines in steroidogenesis. The lack of release of cyclic AMP from the tissue after noradrenaline stimulation is unexpected in the light of the increases in medium levels of cyclic AMP observed after incubation of follicles with gonadotrophins. The significance of the differences in the pattern of cyclic AMP increase in the tissue and medium during incubation of follicles with noradrenaline, LH or FSH remains to be ascertained. They may be due to differences in the site of action of the three hormones or to different effects on tissue phosphodiesterase activity. A better understanding of the role of catecholamines in the ovary will be gained by studying the effect of specific α and β agonists and antagonists on both follicular contractility and ovulation, and on steroidogenesis.

The lack of effect of prolactin on follicular cyclic AMP may reflect a lack of prolactin receptors on the preovulatory follicle. Alternatively prolactin action may not be exerted through a stimulation of adenylate cyclase but through other mechanisms, such as the increase

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in Na+/K+ ATPase sites recently demonstrated in guinea pig myometrium (Lipton, Leah and Parkington, 1978). Prolactin constitutes part of the luteotrophic complex necessary for the maintenance of secretory activity of the corpus luteum in a variety of species including the sheep (Denamur, Martinet and Short, 1973). An increase in binding of prolactin to rat granulosa occurs after LH treatment and appears to be functionally related to the ability of luteal cells to respond to prolactin (Richards and Williams, 1976). While prolactin's role is largely regarded as luteotrophic, it may have a regulatory function in the preovulatory follicle. In the human, at least, low levels of prolactin are necessary for progesterone secretion by cultured granulosa cells while high levels inhibit progesterone secretion and block the action of LH or FSH (McNatty, Sawers and McNeilly, 1974). Whether or not prolactin has a role in the large follicle in the sheep, remains to be determined.

CHAPTER 4

MATURATIONAL CHANGES IN SHEEP OVARIAN FOLLICLES: GONADOTROPHIC STIMULATION OF CYCLIC AMP PRODUCTION BY ISOLATED THECA AND GRANULOSA CELLS

4.1 INTRODUCTION

Until recently one of the major impediments to our understanding of ovarian function was the lack of an experimental system in which homogenous ovarian cell populations could be examined. The ovary is a heterogenous organ with a number of compartments able to respond differently to gonadotrophins, making interpretation of results from studies on whole ovaries or ovarian slices difficult. In the preceding chapter a method has been described for examining the response of isolated follicles freed from the contamination of stroma and luteal cells. The follicle, however, consists of two main cell types, the theca and membrana granulosa. An indication that these cells respond differently to gonadotrophins was first obtained from autoradiographic studies which showed that LH binding (I^{125}-hCG) occurred predominantly in the theca cell layer (Rajaniemi and Vanha-Perttula, 1972; Zeleznik et al. 1974) while FSH binding was associated more with the granulosa (reviewed by Channing and Kammerman, 1974).

More recent evidence suggests that the pre or neonatal ovary does not contain any LH binding sites (Siebers and Engel, 1977). Binding sites for hCG appear in rat ovaries during the first post natal week (Kolena, 1976), and by 25 days of age binding sites for both hCG and FSH can be demonstrated in the theca and granulosa respectively (Zeleznik et al. 1974). FSH induces the appearance of LH receptors in rat (Zeleznik et al. 1974) and pig granulosa cells (Channing, 1975) and

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increases aromatase activity in rat granulosa cells (Armstrong and Dorrington, 1977). The number of LH or hCG receptors or at least the ability to bind labelled gonadotrophins, increases with maturation in pig granulosa cells (Channing and Kammerman, 1973, 1974; Kammerman and Ross, 1975; Nakano et al. 1977) and is associated with an increase in adenylate cyclase activity (Lee, 1976). Incubation of rat ovarian fragments with FSH in vitro results in increased binding of ^{125}I-hCG to granulosa cells (Nimrod et al. 1977).

4.2 AIM

This study was undertaken, firstly to establish the intrafollicular site(s) of action of LH and FSH. Secondly, to see if the changes in the capacity of maturing granulosa cells to bind ^{125}I-hCG are reflected in the ability of gonadotrophins to stimulate cyclic AMP production in theca and granulosa preparations isolated from follicles of different sizes.

4.3.1 Experiment 1 (a)

Aim: To determine the relative contribution of the theca and granulosa to cyclic AMP levels previously measured in the whole follicle.

Culture Conditions: In this study large follicles (4 - 6 mm) were dissected from the ovaries of sheep between Days 4 - 14 of the cycle and incubated for 18 h in gonadotrophin free medium under the conditions described by Moor et al. (1973) and in Chapter 2. Following the overnight incubation the follicles were cultured for a further 40 min under the same conditions but in culture medium containing hCG (20 i.u./ml) or FSH (50 µg/ml) or no gonadotrophin and 8 mM theophylline.

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Isolation of Theca and Granulosa: At the end of the 40 min each follicle was placed in 1 ml of ice-cold incubation buffer containing 8 mM theophylline, and a small incision made in the follicle wall. The granulosa cells and follicular fluid were washed from the thecal casing using an angled Pasteur pipette with a tip drawn to a 0.5 mm point. The washing process was repeated 15 times to ensure nearly complete removal of granulosa cells. (Histological examination of washed theca revealed that invariably a small number of granulosa cells remained attached to the theca). The thecal casing was transferred to a second vessel containing 1 ml ice-cold buffer. The separated tissues were homogenized directly in the buffer and after centrifugation the supernatant was assayed for cyclic AMP (as described in Chapter 2). Results are expressed as pmol/mg wet wt.

Results: In the *control follicle 80% of the cyclic AMP was associated with the theca. However, when the follicles were incubated for 40 min with hCG (20 i.u./ml) for FSH (50 µg/ml) there was an increase in cyclic AMP levels, predominantly in the granulosa cells (Fig. 4.3.1 b). Both hCG and FSH increased cyclic AMP levels in the granulosa while FSH, but not hCG, significantly increased levels in the theca (Table 4.3.2).

4.3.2 Experiment 1 (b)

Aim: To examine the cyclic AMP production by theca and granulosa cells separated before treatment with gonadotrophin.

* control follicles incubated for 40 min without hormone

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Table 4.3.1: Effect of FSH (50 µg/ml) and hCG (20 i.u./ml) on cyclic AMP levels in theca and granulosa, separated prior to a 40 min incubation with gonadotrophin.

Treat.	Theca m	Theca 95% conf. l.	Theca $\bar{R}$	Granulosa m	Granulosa 95% conf. l.	Granulosa $\bar{R}$	N
cont.	3.2	(1.9 - 6.8)	8.2	1.1	(0.6 - 1.9)	13.6	10
FSH	8.4	(1.0 - 16.6)	12.4	1.4	(0.2 - 1.9)	14.7	9
hCG	35.5	(23.2 - 47.8)	26.5	2.2	(1.1 - 6.2)	22.2	12

The following pairs of groups are significantly different at the P = 0.05 level:

control and hCG	none
FSH and hCG	

Table 4.3.2: Effect of FSH (50 µg/ml) and hCG (20 i.u./ml) on cyclic AMP levels in theca and granulosa, separated following a 40 min incubation with gonadotrophin.

	Theca			Granulosa			
cont.	3.1	(2.4 - 7.7)	16.3	0.7	(0.1 - 1.3)	11.1	20
FSH	7.9	(4.6 - 10.1)	31.4	26.8	(11.6 - 59.1)	33.6	5
hCG	9.9	(6.1 - 16.8)	24.7	36.2	(6.3 - 53.8)	30.2	17

The following pairs of groups are significantly different at the P = 0.05 level:

control and FSH	control and hCG
	control and FSH

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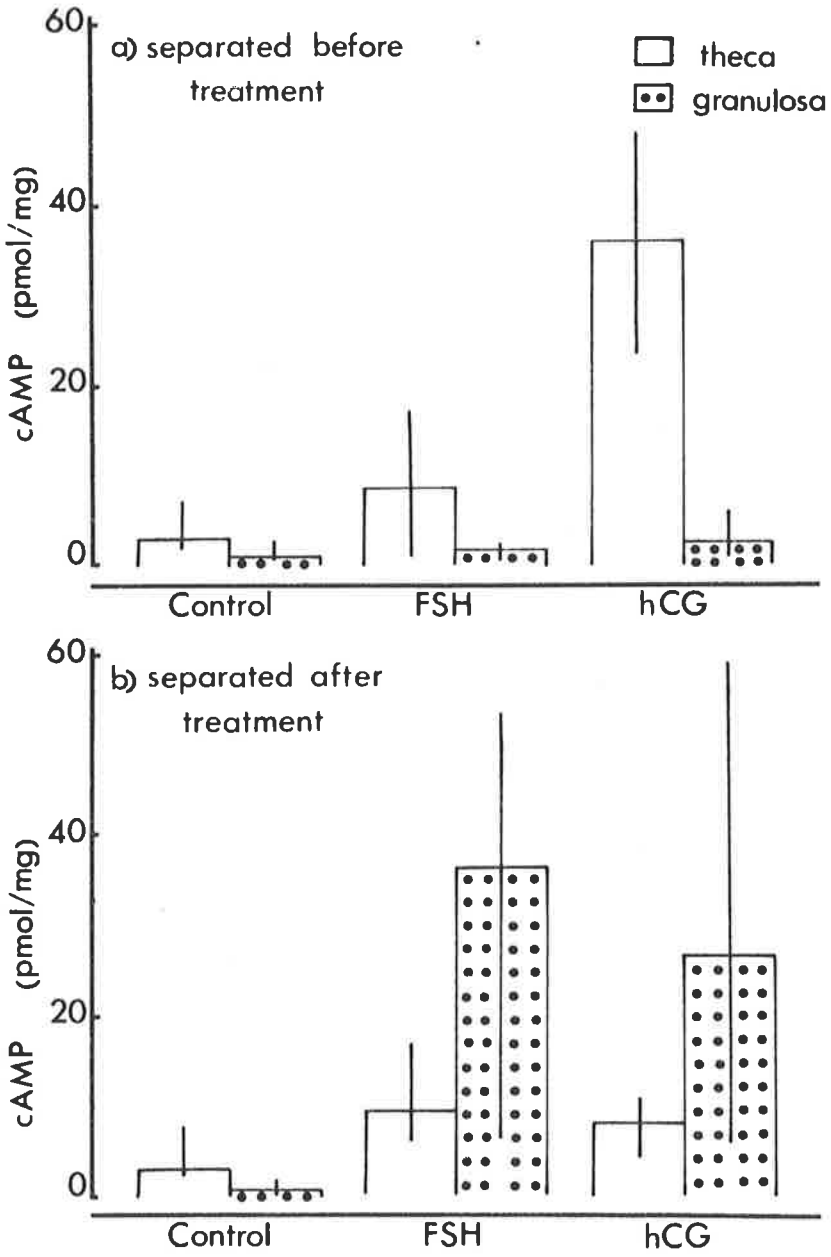

Figure 4.3.1 Effect of FSH (50 ug/ml) and hCG (20 i.u./ml) on cyclic AMP levels in theca and granulosa cell preparations separated before and after a 40 min incubation.

Values plotted are medians (95% conf. limits). See Tables 4.3.1 and 4.3.2 for details.

Culture Conditions: The theca and granulosa cells were prepared and separated as described in Exp. 1 (a). The theca and granulosa cells were separately incubated in 1 ml of incubation buffer containing the appropriate gonadotrophin (and under the same conditions described for the overnight incubation). After 40 min the tissue was homogenized in ice-cold incubation buffer and assayed for cyclic AMP as described earlier. Results are expressed as pmol/mg wet wt.

Results: The results are shown in Fig. 4.3.1a. Both hCG and LH (50 µg/ml; data not shown in Fig.), but not FSH, significantly increased the cyclic AMP level in the theca. None of the treatments resulted in an increase in cyclic AMP levels in the granulosa cells.

A comparison of the cyclic AMP levels in the theca and granulosa following incubation of intact follicles or separated tissue, with gonadotrophin, is presented in Table 4.3.3.*

Table 4.3.3: Comparison of cyclic AMP levels in theca and granulosa following incubation of intact or separated tissue with gonadotrophin.

Treatment	Theca	Granulosa	Total
control	N.S.	N.S.	N.S.
hCG	intact < sep.	intact > sep	N.S.
	$p < 0.001$	$p < 0.0004$	
FSH	N.S.	intact > sep.	intact > sep.
		$p < 0.01$	$p < 0.01$

Separation of the theca and granulosa cells before or after the 40 min incubation did not alter the cyclic AMP level in the control groups.

* i.e. A comparison of the method of separation on the response to FSH and hCG (see Figure 4.3.1).

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Following an 18 h culture

Separation of the tissue before treatment did not alter the effect of FSH on the theca but reduced its effect on cyclic AMP levels in the granulosa cells. In contrast, cyclic AMP levels were higher in the theca and lower in the granulosa cells of follicles separated before treatment with hCG.

The finding that isolated granulosa cells failed to respond to either hCG or FSH was surprising, particularly in view of the report of gonadotrophin receptors associated with granulosa cells. This study was then extended with the method modified so that granulosa cells were incubated in the absence of follicular fluid.

4.4 EXPERIMENT 2

Aim: Firstly, to investigate the responsiveness of isolated theca and granulosa cells incubated in the absence of follicular fluid. Secondly, to see if changes in the capacity of maturing granulosa cells to bind hCG are reflected in the ability of gonadotrophins to stimulate cyclic AMP production in theca and granulosa cell preparations isolated from follicles of different sizes.

Isolation of theca and granulosa: In this study follicles between 1 and 3 mm and 4 and 6 mm in diameter were dissected from ovaries of sheep between Days 4 and 14 of the cycle. Follicles in the two size classes were pooled and selected at random for use in the control and treatment groups.

Each follicle was cut almost in half with a fine pair of dissecting scissors, and the granulosa cells removed by gently scraping the inside of the follicle with a curved glass probe. The granulosa

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cells were initially suspended in Dulbecco-phosphate buffer containing 4 mM EDTA, which was found to be essential to prevent the follicular fluid (particularly from large follicles) from clotting. In each experiment, the cells from up to 40 small follicles or 10 - 15 large follicles were pooled and centrifuged at 50 - 60 g for 5 min (unlike experiment 1, where each follicle was incubated separately). They were then washed twice in EDTA free buffer, suspended in an appropriate volume of HEPES-buffered Medium 199 containing 8 mM theophylline and 0.5 ml fractions were distributed amongst the various control and treatment groups. The scraped thecal tissue was cut into several pieces, washed with Dulbecco-phosphate buffer and distributed similarly. Histological examination of the theca usually revealed some granulosa cells remaining attached to the basement membrane. The granulosa cell suspensions invariably contained a disappointingly large number of broken cells. These probably result from the separation of cells which have interconnecting cytoplasmic processes which are a feature of the cells in the basal region of the membrana granulosa (Hay and Moor, 1975).

In order to reduce the effect of the variation in yield of cells between experiments, where possible, matched or related sample statistical tests were used to analyse the results.

Culture Conditions: The incubation of theca and granulosa cells was carried out in a shaking water bath at 37°. Incubations were begun by the addition of a small volume of medium containing either FSH or hCG to give a final concentration of 5 µg/ml and 5 i.u./ml respectively, and ended after 40 min by rapid freezing.

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The processing of the tissue samples was modified slightly from that used in the preceding experiments. Prior to assay the theca and granulosa cell preparations were thawed and broken up by sonication (Sonifer, B-12, Branson Sonic Power Co., Danbury, Connecticut, U.S.A.). Protein was precipitated by the addition of 3 volumes of ethanol and protein-free supernatant was assayed for cyclic AMP. No attempt was made to assay the medium and tissue separately. Protein was determined in the precipitates by the method of Lowry, Rosebrough, Farr, Randall (1951), using bovine serum albumin as standard. Results are expressed as pmol cyclic AMP/mg protein.

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Results: The medians and 95% confidence limits for cyclic AMP production by theca and granulosa cell preparations after treatment with FSH and hCG are presented in Table 4.4.1. Summaries of statistical analyses of these data are presented in Tables 4.4.2 a, b and c.

Table 4.4.1: Effect of FSH and hCG on cyclic AMP formation by theca and granulosa preparations.

		cAMP (pmol/mg protein)		
Tissue Type	Follicle Size	Control	FSH (5 µg/ml)	hCG (5 i.u./ml)
Theca	Large (4 - 6 mm)	3.4[1] (0 - 9,8)[2]	16.7 (5.8 - 53.0)	152.3 (141.1 - 300)
	Small (1 - 3 mm)	0.26 (0 - 7.8)	17.2 (0 - 25.8)	157.8 (38.7 - 280)
Granulosa	Large	3.9 (0 - 11.0)	17.1 (9.4 - 133)	42.7 (11.4 - 268)
	Small	8.6 (0 - 10.2)	75.2 (19.6 - 268)	3.6 (0 - 16.2)

1. Median
2. 95% confidence limits

Effect of hCG and FSH on theca and granulosa from large follicles: In contrast to the results of experiment 1 (b) hCG increased cyclic AMP in both theca and granulosa cells from large follicles when incubated in the absence of follicular fluid. FSH increased cyclic AMP levels in the granulosa, but not to the same extent as hCG, and, unlike hCG, did not stimulate levels in the theca.

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Effect of hCG and FSH on theca and granulosa from small follicles: Thecal cells from small follicles responded in a similar fashion to those from large follicles; hCG increased cyclic AMP levels while FSH was without effect. The granulosa cells from small follicles on the other hand, responded quite differently to those from large follicles. The levels of cyclic AMP in the granulosa cells of small follicles were higher after exposure to FSH than after either hCG or no treatment. Unlike the response of the granulosa cells from large follicles there was no difference between the hCG treated group and the control group.

Comparison of responses of large and small follicles: The theca from large and small follicles produced a very similar increase in cyclic AMP levels after exposure to hCG. In contrast, it was apparent that the most dramatic changes in hormone responsiveness were occurring in the granulosa, since the development from a 1 - 3 mm to a 4 - 6 mm follicle resulted in an increased cyclic AMP response to hCG and a reduced cyclic AMP response to FSH.

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Table 4.4.2: Summaries of statistical analyses of results.

Presented in Table 4.4.1, expressed as P values.

a) Effect of gonadotrophin

Large follicle granulosa			Large follicle theca		
	Control	FSH		Control	FSH
FSH	.05[1]		FSH	NS	
hCG	.01	NS[2]	hCG	.01	.01

Small follicle granulosa			Small follicle theca		
	Control	FSH		Control	FSH
FSH	.01		FSH	NS	
hCG	NS	.01	hCG	.01	.01

b) Comparison of cell types

	Control	FSH	hCG
Large	.439[3]	.132	.189
Small	.072	.024	.001

c) Comparison of follicle size

	Control	FSH	hCG
Theca	.378[4]	.631	.949
Granulosa	.608	.071	.0004

1. Probability value (Friedman's test) arising from the comparison of control and FSH treatment groups of granulosa cells from large follicles. The other values in a) are to be interpreted in a similar way.

2. Not significant.

3. Probability value (Wilcoxon's test) arising from the comparison of granulosa and theca from large follicles in the control group. The other values in b) are to be interpreted in a similar way.

4. Probability value (Wilcoxon's test) arising from the comparison of theca from large and small follicles in the control group. The other values in c) are to be interpreted in a similar way.

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4.5 DISCUSSION

These results indicate that the hormone responsiveness of the sheep granulosa cell layer undergoes maturational changes similar to cells of pigs (Channing and Kammerman, 1974; Nakano et al. 1977) and the rat (Zeleznik et al. 1974; Richards et al. 1976). Granulosa cells from small follicles respond to FSH but not to hCG, whereas in large follicles hCG is more effective than FSH in stimulating cyclic AMP production. This is in contrast to the results of Experiment 1 which suggest that the granulosa cells from large follicles did not respond to LH or FSH with increased cyclic AMP levels. Although the explanation for this is uncertain, one possibility is that in the former study granulosa cells from each follicle were incubated in the presence of clotted follicular fluid, while in Experiment 2, pooled granulosa cells were incubated after removal of the follicular fluid. The possibility that cyclic AMP production by isolated granulosa cells was inhibited by the presence of clotted follicular fluid is suggested by the finding that the effect of both hCG and FSH on cyclic AMP levels in granulosa cells was significantly reduced if the tissues were separated before treatment. Further evidence of an inhibitory effect of follicular fluid on LH stimulation of cyclic AMP levels in porcine granulosa cells is reviewed by Channing, Anderson and Batta (1978).

The small stimulatory effect of FSH on the cyclic AMP levels in the theca preparation in Experiment 1 was not apparent in Experiment 2 and could be due either to less efficient removal of granulosa cells in Experiment 1 or to LH contamination of the FSH preparation, as the dose of FSH used in Experiment 1 was 10 times that used in Experiment 2 (50 µg/ml compared to 5 ug/ml). The finding that the theca concentration

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of cyclic AMP was significantly greater in the tissue separated before treatment with hCG than in the tissue separated after treatment (Experiment 1, Table 4.3.3) is interesting and cannot be accounted for by the inclusion of cyclic AMP in the medium, as it contributed less than 4 pmol/mg (Fig. 3.4.2). If the difference is not a result of the method of preparation then one possible interpretation is that cyclic AMP produced in the theca as a result of LH stimulation, is released extracellularly and escapes into the granulosa and follicular fluid. Alternatively the theophylline included in the incubation medium may not be able to act as efficiently in the whole follicle as in the separated tissue resulting in a more rapid degradation of cyclic AMP in the theca of the intact follicle.

In Experiment 2 the effect of FSH on the cyclic AMP levels in the granulosa cells from large follicles is reduced when compared to the response of the granulosa cells from small follicles. This is not surprising as it has been shown that granulosa cells of small porcine follicles bind more ^{125}I-FSH than cells of medium or large follicles. The finding that the granulosa cells acquire responsiveness to hCG in the larger follicles is also reflected in binding studies as ^{125}I-LH is bound to a much greater extent by granulosa cells of large follicles (Nakano et al. 1977). More recently, Hamberger, Nordenstrom, Rosberg and Sjogren (1978) have found that rat granulosa cells prepared from small and medium sized follicles increase cyclic AMP levels in response to FSH but not LH, while in cells from preovulatory follicles both gonadotrophins had a stimulatory effect.

In contrast, incubation of thecal tissue from large and small follicles resulted in a similar increase in cyclic AMP levels suggesting

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that the theca contains competent LH receptors at an early stage of development. Furthermore, follicles from both size classes produce comparable amounts of androgen (Chapter 8, this thesis), indicating that the theca is capable of significant steroidogenesis at an early stage.

Turnbull et al. (1977) estimated that four days are required for a 1 - 2 mm sheep follicle to develop into a 4 - 6 mm pre-ovulatory follicle. It is clear that important changes are taking place, particularly in the granulosa of the developing follicle during this period. The mechanism by which LH receptors appear in the granulosa is not known, although from investigations by Richards et al. (1976), with hypophysectomized rats, it appears certain that FSH and oestradiol play a role. The finding that the theca is responsive to LH at an early stage of development suggests the possibility that cyclic AMP of the thecal origin may also influence the development of LH receptors in granulosa, particularly in view of the quantities of cyclic AMP produced by the target cells in the theca, after LH stimulation. Since LH stimulates a considerable release of cyclic AMP from intact follicles (Chapter 3, this thesis), it is likely that the cyclic AMP produced in the theca of small follicles is released into the extracellular space, and is potentially able to influence the development of the granulosa. Whether the theca is a source of cyclic AMP, of aromatizable substrate, or of some other unidentified factor, it seems that its presence, or the presence of some other ovarian component, is required for the successful induction of LH receptors in the granulosa (Nimrod et al. 1977).

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CHAPTER 5

SUPPRESSION OF FOLLICULAR CYCLIC AMP AND STEROID RESPONSE TO GONADOTROPHINS BY PRE-TREATMENT WITH GONADOTROPHINS

5.1 INTRODUCTION

The mechanisms which regulate a biological response to a hormonal stimulus are not clearly understood. Until recently it was thought that the principle regulatory mechanism was the alteration of the concentration of hormone able to interact with the receptor. However, it now seems likely that another, perhaps as important, control mechanism involves the alteration, by the hormone, of the number of its own receptors or that of another hormone (Tata, 1975). This type of regulation has been suggested by evidence that elevated levels of drugs or hormones lead to a desensitization of the target tissues to the specific pharmacological or biochemical effects of these agents (Mukherjee, Caron and Lefkowitz, 1976; Hsueh, Dufau and Catt, 1976; Conti, Harwood, Dufau and Catt, 1977a).

A loss of responsiveness of the ovary to luteinizing hormone (LH) following initial stimulation by the hormone was first reported by Armstrong, O'Brien and Greep (1964). They found that luteinized rat ovaries failed to increase progesterone synthesis when cultured in the presence of LH if they had been previously exposed to LH *in vivo*.

Related studies in the rabbit have shown that while follicles isolated from oestrous rabbits accumulate cyclic AMP after stimulation by LH (Marsh *et al*. 1972), preovulatory follicles (Marsh, Mills and Le Maire, 1973) or follicles obtained from rabbits pretreated with exogenous LH (Le Maire, Davies and Marsh, 1976; Hunzicker-Dunn and Birnbaumer, 1976; Younglai, 1977) become refractory to further stimulation by LH.

In an earlier *in vitro* study, Lamprecht, Zor, Tsafriri and Lindner (1973) found that isolated preovulatory rat follicles pretreated

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with LH did not increase cyclic AMP formation in response to a second exposure to LH. Similarly, pretreatment with FSH and PGE_2 induces refractoriness of preovulatory rat follicles to a second exposure of hormone (Zor, Lamprecht, Misulovin, Koch and Lindner, 1976). A loss of responsiveness of whole rat ovaries (Selstam et al. 1976) or granulosa cells (Richards, Ireland, Rao, Bernath, Midgley and Reichert, 1976) also results from pre-exposure to LH or FSH. More recently, Nilsson, Hillensjo and Ekholm (1977) showed that the endogenous gonadotrophin surge prior to ovulation not only stimulates cyclic AMP formation by the rat ovarian follicle but also results in a desensitization to further stimulation.

5.2 AIM

In the light of these studies we have investigated the effect of pre-exposure of small and large sheep follicles to FSH and hCG on the subsequent cyclic AMP response of isolated theca and granulosa cell preparations to a second incubation with gonadotrophins. Secondly, the testosterone and androstenedione secretion by intact follicles and isolated theca preparations was examined after pre-incubation with hCG. In the third section an attempt was made to overcome the hCG induced inhibition of thecal androgen production by incubating the tissue with dibutyryl cyclic AMP.

5.3 Experiment 1

Aim: The aim of these experiments was to investigate the effect of pre-exposure of small and large sheep follicles to FSH and hCG on the subsequent cyclic AMP response of isolated theca and granulosa cell preparations to a second incubation with gonadotrophin.

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Culture Conditions: Follicles between 1 and 3 mm and 4 and 6 mm in diameter were dissected from the ovaries of sheep between Days 4 and 14 of the cycle. Follicles in the two size classes were pooled and selected at random for use in the control and treatment groups. Each experiment was repeated on at least 4 occasions. On each occasion the theca and granulosa cells were isolated from 40 - 60 small follicles or 20 - 30 large follicles dissected from the ovaries of 20 - 30 sheep.

The whole follicles were incubated for 18 h in the presence of hCG (1 i.u./ml) or FSH (1 ug/ml) under the conditions described by Moor et al. (1973).

Following the overnight incubation the follicles were washed and separated into theca and granulosa cell fractions as described in Chapter 4.5. The incubation of theca and granulosa cells was carried out in a shaking waterbath at 37°. Incubations were begun by the addition of a small volume of medium containing either FSH or hCG to give a final concentration of 5 ug/ml or 5 i.u./ml respectively, and ended after 40 min rapid freezing.

Prior to assay the tissue was sonicated and cyclic AMP and protein determined as described in Chapter 4.5. Results are expressed as pmol cyclic AMP/mg protein.

Results: In order to simplify the description of the groups they are identified by a two-part code. The first part of the code represents the pre-treatment and the second part the treatment period. Control-hCG therefore describes an 18 h incubation of intact follicles in gonadotrophin free medium followed by a second incubation in fresh medium containing hCG. In Experiment 1, the duration of the second incubation was 40 min.

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The medians and 95% confidence limits are plotted in Figures 5.3.1 and 2. Probability values for the results in the figures are given in Table 5.3.1.

Cyclic AMP Levels in Isolated Theca and Granulosa Cells Following Control-hCG and Control-FSH Treatment: In this study the response of tissues isolated from both large (4 - 6 mm) and small (1 - 3 mm) follicles was examined.

Both hCG and FSH stimulated cyclic AMP levels in the granulosa cells from large follicles (Fig. 5.3.1b) while the theca from large follicles responded to hCG but not to FSH (Fig. 5.3.2b). FSH significantly increased the level of cyclic AMP in the granulosa cells from small follicles while hCG had no effect (Fig. 5.3.1a). The theca from the small follicles, like that from the large, responded only to hCG (Fig. 5.3.1b).

While the pattern of response of the tissues from small and large follicles was the same as that found previously (Table 4.5.1) the amount of cyclic AMP produced by the tissues separated after an 18 h preincubation differ from those found in tissues treated without the preincubation. It is probably unwise to make too much of the differences, due to the relatively large between-experiment variability mentioned earlier (Ch. 4.5). However, the 18 h control incubation caused a reduction in the cyclic AMP production by the theca of follicles of both size classes in response to hCG but increased the response of the granulosa cells from small and large follicles to FSH, and FSH and hCG, respectively.

Cyclic AMP Levels in Isolated Theca and Granulosa Following hCG-hCG and hCG-FSH Treatment: Preincubation with hCG significantly reduced the response of granulosa cells from large follicles (Fig. 5.3.1b) and theca

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from large and small follicles (Fig. 5.3.1a and b) to a second exposure to hCG. The response of granulosa cells from large follicles to FSH was significantly reduced by pretreatment with hCG (Table 5.3.1a), although not to the same extent as the hCG response. The FSH induced increase in cyclic AMP levels in granulosa cells from small follicles was not significantly reduced by hCG pretreatment.

Cyclic AMP Levels in Isolated Theca and Granulosa Cells Following FSH-hCG and FSH-FSH Treatment: The granulosa cells from both large and small follicles showed an 80% reduction in cyclic AMP levels after FSH stimulation when prepared from follicles pretreated with FSH compared with those prepared from control follicles (Fig. 5.3.1). Pretreatment with FSH also reduced the hCG induced rise in cyclic AMP levels in the granulosa from large follicles and in the theca from large and small follicles. However, this decline in responsiveness to hCG was not as great as that to FSH.

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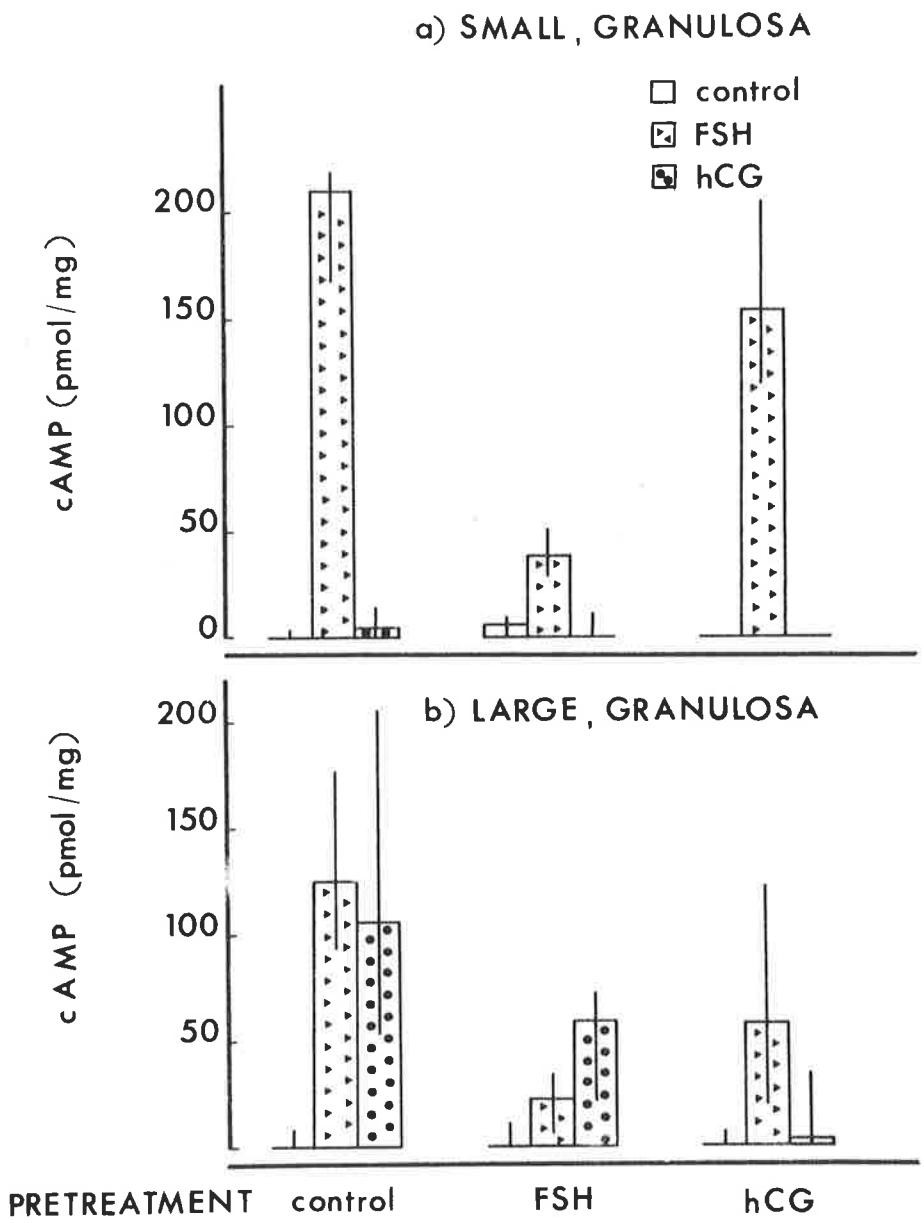

Figure 5.3.1 Cyclic AMP levels in granulosa cells isolated from small (a) and large (b) follicles, following a 40 min incubation in control medium, or medium containing FSH (5 ug/ml) or hCG (5 i.u./ml). Prior to separation into theca and granulosa the intact follicles were incubated for 18 h in control medium or medium containing FSH (1 ug/ml) or hCG (1 i.u./ml).

Results are expressed as medians; vertical bars represent 95% confidence limits. A summary of the statistical analysis is given in Table 5.3.1.

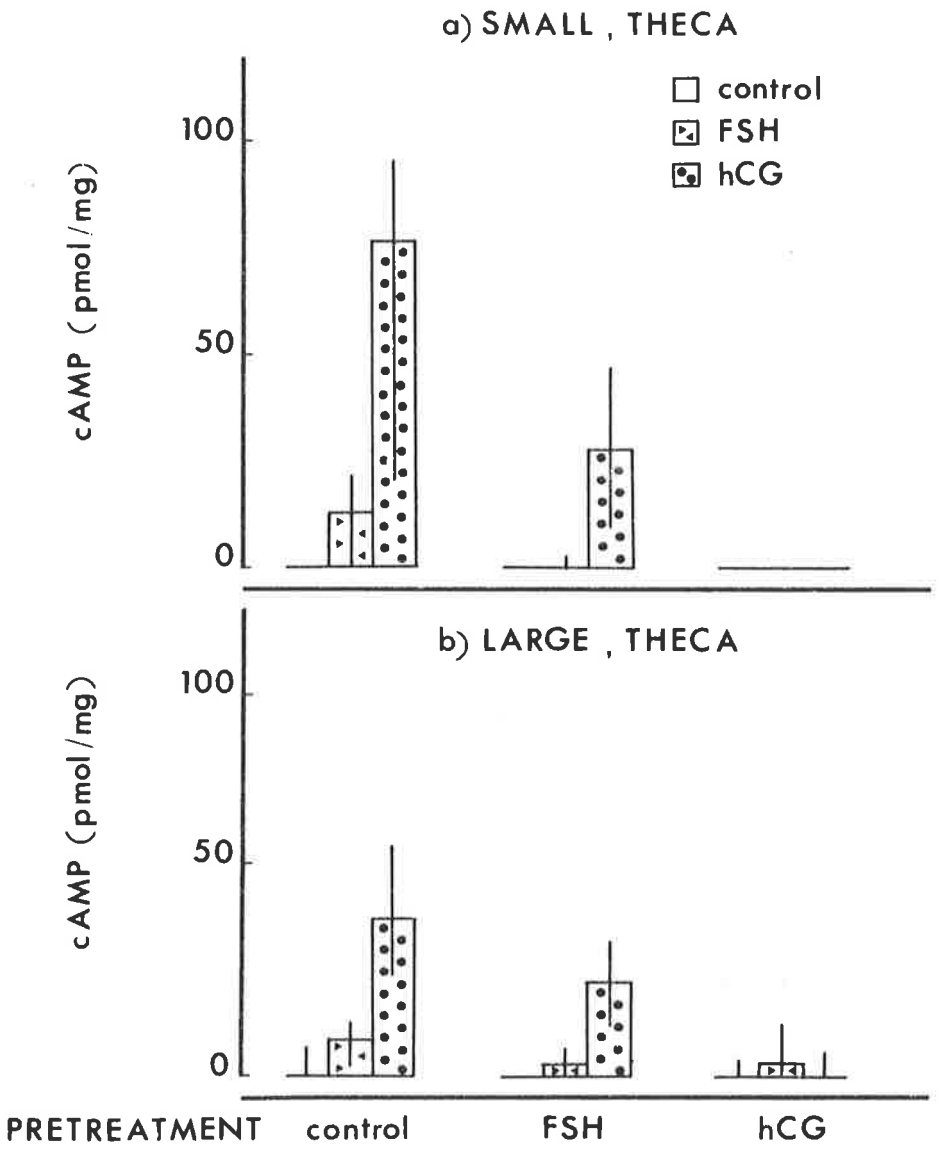

Figure 5.3.2 Cyclic AMP levels in thecal tissue isolated from small (a) and large (b) follicles, following a 40 min incubation in control medium or medium containing FSH (5 ug/ml) or hCG (5 i.u./ml). See legend to Figure 5.3.1 for experimental details.

Table 5.3.1: Summaries of statistical analyses of results presented in Figs. 1 - 3, expressed as P values.

		Cont-cont		Cont-hCG		Cont-FSH
(a) Effect of Pretreatment						
Small follicle, granulosa	hCG-cont	1[1]	hCG-hCG	1	hCG-FSH	0.779
	FSH-cont	0.075	FSH-hCG	0.110	FSH-FSH	0.005
Small follicle, theca	hCG-cont	1	hCG-hCG	0.043	hCG-FSH	1
	FSH-cont	0.180	FSH-hCG	0.007	FSH-FSH	0.012
Large follicle, granulosa	hCG-cont	0.593	hCG-hCG	0.003	hCG-FSH	0.015
	FSH-cont	1	FSH-hCG	0.003	FSH-FSH	0.002
Large follicle, theca	hCG-cont	0.463	hCG-hCG	0.002	hCG-FSH	0.155
	FSH-cont	0.225	FSH-hCG	0.028	FSH-FSH	0.004

		Cont-cont	Cont-hCG		hCG-cont	hCG-hCG		FSH-cont	FSH-hCG
(b) Effect of Treatment									
Small follicle, granulosa	Cont-hCG	NS[2]		hCG-hCG	NS		FSH-hCG	NS	
	Cont-FSH	0.01[3]	0.01	hCG-FSH	0.02	0.02	FSH-FSH	0.01	0.01
Small follicle, theca	Cont-hCG	0.01		hCG-hCG	NS		FSH-hCG	0.01	
	Cont-FSH	NS	0.01	hCG-FSH	NS	NS	FSH-FSH	NS	0.01
Large follicle, granulosa	Cont-hCG	0.01		hCG-hCG	NS		FSH-hCG	0.01	
	Cont-FSH	0.01	NS	hCG-FSH	0.01	0.01	FSH-FSH	NS	0.01
Large follicle, theca	Cont-hCG	0.01		hCG-hCG	NS		FSH-hCG	0.01	
	Cont-FSH	NS	0.05	hCG-FSH	NS	NS	FSH-FSH	NS	NS

Table 5.3.1: Summaries of statistical analyses of results presented in Figs. 1 - 3, expressed as P values cont.

		Cont-cont		hCG-hCG
Testosterone	Cont-hCG hCG-cont	0.035[4] 0.0012	Cont-hCG hCG-cont	0.0016 0.068
Androstenedione	Cont-hCG hCG-cont	0.055 0.0002	Cont-hCG hCG-cont	0.0027 0.048

1. Probability value (Wilcoxon matched-pairs signed-ranks test) arising from the comparison of control-control and FSH-control treatment groups of granulosa cells from small follicles. The other values in a) are to be interpreted in a similar way.
2. Not significant. A P value of 0.05 or less is considered to indicate a significant difference.
3. Probability value (Friedman's test) arising from the comparison of control-control and control-FSH treatment groups of granulosa cells from small follicles. The other values in b) are to be interpreted in a similar way.
4. Probability value (Mann-Whitney U test) arising from the comparison of the testosterone production by control-control and control-hCG treatment groups of intact small follicles. The other values in c) are to be interpreted in a similar way.

5.4 Experiment 2

Aim: In this study an attempt has been made to discover the consequences of desensitization of follicular adenylate cyclase to androgen production by intact small follicles.

Culture Conditions: The follicles (1 - 3 mm) were prepared as described for Experiment 1. At the end of the 18 h preincubation with or without hCG (1 i.u./ml) the medium was replaced with fresh medium containing hCG (5 i.u./ml) or no treatment. The follicles were then incubated again under the same conditions as those described for the 18 h incubation. The medium and follicles were harvested after 6 h.

Androstenedione and testosterone were assayed by validated radioimmunoassay procedures previously described by Janson et al. (1978) and in Chapter 2 (this thesis).

Results:

Androstenedione and Testosterone Secretion by Intact Small Follicles.

Effect of hCG Pretreatment: In the control - hCG group, that is the follicles that had not been pretreated with hCG but had been exposed to hCG (5 i.u./ml) during the 6 h treatment period, testosterone secretion into the medium was greater than that of the control-control group. (Fig. 5.3.3.) In contrast, the group pretreated with hCG failed to respond to the second treatment. Androstenedione levels were slightly increased in both the control-hCG and hCG-hCG compared to the control-control and hCG-control groups respectively. However, preincubation with hCG significantly reduced androstenedione and testosterone secretion by control and hCG treated follicles compared to the control pretreatment groups.

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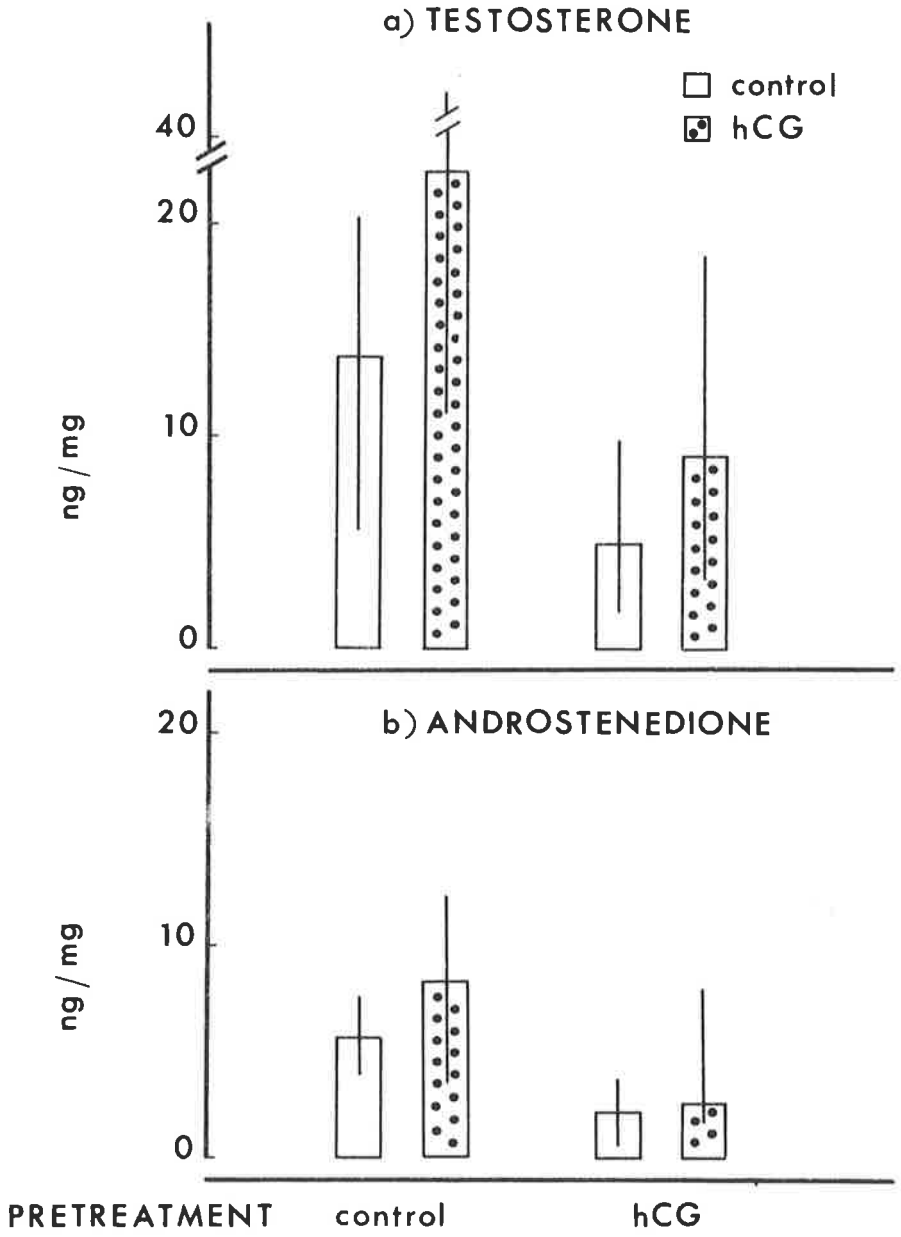

Figure 5.3.3 Testosterone and androstenedione production by intact small follicles following a 6 h incubation in control medium or medium containing hCG (5 i.u./ml). The follicles were previously incubated for 18 h in control medium or medium containing hCG (5 i.u./ml).

Results are expressed as medians; vertical bars represent 95% confidence limits, N = 10. A summary of the statistical analysis is given in Table 5.3.1.

5.5 Experiment 3

Aim: One of the aims of this series of experiments was to see if the hCG induced reduction in androgen secretion by small follicles occurred in large (4 - 6 mm) follicles and after a shorter period of preincubation. The effect of hCG on the secretion of androgens from sheep theca and follicle wall was therefore investigated after varying periods in culture.

Secondly, since it seemed likely that the inability of hCG to stimulate androgen production after pretreatment with hCG resulted from a desensitization of follicular adenylate cyclase, the effect of exogenous dibutyryl cyclic AMP on theca androgen secretion has been examined.

Culture Conditions: In these experiments two tissue preparations were used; the theca and theca plus granulosa(i.e.follicle wall) from large (4 - 6 mm) follicles. The theca was prepared by the same method as that described in Chapter 4. Where possible pieces of theca were distributed in such a way that each treatment group contained pieces of theca from the same follicles, in an attempt to reduce the effect of between follicle variance. The follicle wall was prepared by carefully cutting the follicles into pieces (usually quarters) without disturbing the granulosa, and distributing the tissue in a similar way to the theca.

Following the separation of the tissue, the theca and follicle wall preparations were placed on stainless steel grids and incubated again in the presence or absence of hCG (5 i.u./ml) under the same conditions as those described for the 18 h incubation. The medium was changed at 6 h intervals and assayed for testosterone and androstenedione. At the end of the incubation the granulosa cells were scraped off the theca of the follicle wall preparations. The theca was then blotted dry and weighed; results are therefore expressed as ng/mg wet wt. of theca.

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Results:

Effect of hCG on Secretion Rate of Androgens After Varying Periods of Culturing Sheep Theca and Follicle Wall Preparations: The median secretion rates of total androgen (androstenedione + testosterone) by the theca and follicle wall are presented in Table 5.5.1 and Fig. 5.5.1. Statistical analyses of the results are presented in Table 5.5.2.

Effect of hCG on the Theca: The addition of hCG to the theca significantly increased androgen secretion during the first 6 h. In contrast androgen SECRETION declined during the second 6 h of culture and still further between 12 and 24 h. This decline was not prevented by the continued presence of hCG in the medium.

Effect of hCG on the Follicle Wall: Like the theca, the follicle wall secreted significantly more androgen in the presence of hCG during the first 6 h of culture. However, androgen secretion declined during the second 6 h of culture and by 12 - 24 h was significantly lower than the control group (Table 5.5.2a).

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Table 5.5.1: Comparison of Androgen (T + A) Secretion (ng/h/mg theca) by Theca and Follicle Wall Incubated With and Without 5 i.u./ml hCG (N = 8).

	Theca		Follicle Wall	
treatment (h)	m	95% conf. limits	m	95% conf. limits
control				
0 - 6	4.3	0.6 - 11.3	7.5	0.3 - 18.3
6 - 12	2.6	0.2 - 5.6	6.4	1.5 - 11.7
12 - 24	1.4	0.1 - 4.4	4.4	0.1 - 9.1
hCG				
0 - 6	9.4	1.2 - 16.9	16.3	9.6 - 23.4
6 - 12	2.1	0.3 - 5.5	5.8	2.8 - 9.0
12 - 24	0.3	0.2 - 1.9	1.1	0.3 - 2.0

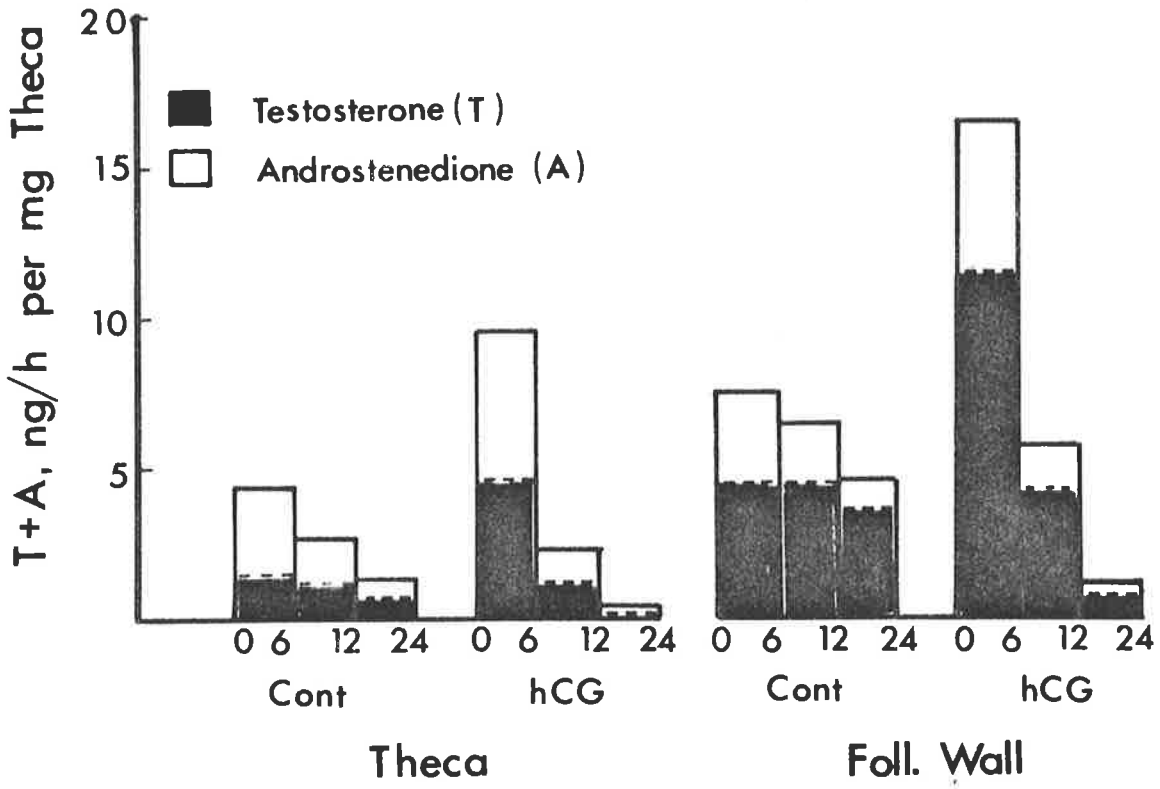

Figure 5.5.1 Effect of hCG (5 i.u./ml) on secretion rate of androgens after varying periods (h) of culturing sheep theca and follicle wall preparations.

Results are expressed as medians. See Table 5.5.1 for further details.

Table 5.5.2: Statistical analysis of the results presented in Figure 5.5.1.

a) Comparison of the effects of hCG and control treatment on androgen production (T + A) by theca and follicle wall.

T[1]	Theca		Follicle Wall	
0 - 6	hCG > cont.	(0.05)[2]	hCG > cont.	(0.01)
6 - 12	N.S.	(0.46)	N.S.	(0.17)
12 - 24	N.S.	(0.14)	hCG < cont.	(0.01)

b) Comparison of androgen (T + A) production of the theca and follicle wall.

T	Control		hCG	
0 - 6	N.S.	(0.14)	F.W. > theca	(0.02)
6 - 12	F.W. > theca	(0.01)	F.W. > theca	(0.01)
12 - 24	F.W. > theca	(0.02)	F.W. > theca	(0.03)

c) Comparison of androgen (T + A) production during each of the incubation periods.

Androgen production was significantly lower ($P < 0.01$[3]) during the 12 - 24 h period than during the 0 - 6 h period in each of the groups; that is, control-theca, hCG-theca, control-follicle wall and hCG-follicle wall.

1. T = Length of incubation (hours).
2. P = Probability value obtained using the Mann-Whitney test.
3. P = Probability value obtained using the Friedman analysis of variance.

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Comparison of Androgen Secretion by Theca and Follicle Wall: Perhaps the most interesting finding of this study was the large difference in the androgen production between the theca and follicle wall. During the 6 h culture periods in both control and hCG treated groups, the androgen secreted by the follicle wall was greater than that secreted by the theca (with the exception of the control, 0 - 6 h period which was not significantly different, P = 0.14). Secondly, the ratio of testosterone to androstenedione was greater in the follicle wall secretion than in the equivalent theca preparation (Fig. 5.5.1).

5.6 Experiment 4

The preceding results suggest that hCG has two effects on androgen secretion by the sheep follicle; firstly it stimulates, then it inhibits, androgen production by the theca or follicle wall. In Experiment 1 (5.3) it was found that pre-exposure of the follicle to hCG (or FSH) resulted in a loss of the ability of theca or granulosa cells to synthesize cyclic AMP in response to a second exposure of gonadotrophin. Secondly, androgen production was inhibited and small follicles examined were unable to increase androgen secretion in response to a second exposure to hCG. It therefore seemed likely that the inhibition of androgen secretion observed during the 12 - 24 h culture period in Experiment 3, and the inability of hCG to prevent this decline, may be due to a fall in intracellular cyclic AMP levels resulting from an inability of hCG to continue to stimulate cyclic AMP production.

Aim: The aim of this experiment was to investigate the possibility that the decline in androgen production after hCG stimulation was the result of a desensitization of thecal adenylate cyclase.

Culture Conditions: The tissue was prepared as for Experiment 3, except

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that only thecal tissue was used. The tissue was distributed so that as far as possible the same tissue was represented in each treatment group, permitting the use of related-sample analysis of the results. The four groups were control-control (theca incubated in gonadotrophin free medium for 6 h when the medium was replaced with gonadotrophin free medium and the incubation continued for a further 6 h), hCG-hCG (medium included hCG (5 i.u./ml), hCG-dbc (1 mM) and hCG-dbc (5 mM). The tissue in the latter two groups was incubated for 6 h with hCG (5 i.u./ml), washed and the medium replaced with fresh medium containing 1 or 5 mM dibutyryl cyclic AMP (dbc), without hCG.

At the completion of the incubation the medium was collected and frozen until assayed for testosterone and androstenedione and the theca was blotted dry and weighed. Since there was no difference between the pattern of secretion of testosterone compared to testosterone plus androstenedione (T + A) by the theca, results are expressed as ng (T + A) per mg theca.

Results: Incubation of theca with hCG (5 i.u./ml) resulted in a significant increase in androgen secretion during the first 6 h and a significant decrease during the second 6 h (the combined data for 4 experiments are presented in Table 5.6.1).

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Table 5.6.1: Androgen (T + A) Secretion by Cultured Sheep Theca. The Tissue was Cultured for 12 h and the Medium Collected at 6 h and 12 h. Results are Expressed as Median ng (T + A)/mg Wet Wt. Theca/6 h.

Treatment	Control	Control	hCG	hCG
time	0 - 6	6 - 12	0 - 6	6 - 12
median	36.2	17	51.0	8.2
95% c.l.	22.0 - 47.1	8.7 - 28.4	33.5 - 66.8	5.4 - 16.0
N	18	18	18	18

The following groups are significantly different at the P = 0.01 level (Wilcoxon matched-pairs signed-ranks test).

cont (0 - 6) > cont (6 - 12)

cont (0 - 6) < hCG (0 - 6)

hCG (0 - 6) > hCG (6 - 12)

cont (6 - 12) > hCG (6 - 12)

The incubation of hCG-pretreated theca with 1 mM dbc did not alter the androgen secretion compared with the hCG treated groups (hCG-hCG, 6 - 12 h, Table 5.6.2, Fig. 5.6.2a). A large dose of dbc (5 mM) slightly increased the androgen secretion of the hCG-pretreated theca (Table 5.6.2, Fig. 5.6.1). It was not sufficient, however, to increase androgen secretion to that of the control-control theca.

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Table 5.6.2: Effect of dibutyryl cyclic AMP (dbc) on the decline in androgen secretion by the theca following treatment with hCG (5 i.u./ml).

Treatment	0 - 6 h m	0 - 6 h 95% conf. l.	6 - 12 h m	6 - 12 h 95% conf. l.	N
cont. - cont.	48.3	(19.9 - 62.5)	25.7	(9.1 - 42.9)	8
hCG - hCG	62.5	(30.7 - 87.4)	13.6	(2.6 - 19.2)	8
hCG - dbc (1 mM dbc)	65.3	(35.1 - 88.0)	13.8	(4.0 - 23.3)	8
cont. - cont.	27.0	(14.9 - 44.8)	10.4	(6.0 - 26.7)	10
hCG - hCG	42.0	(29.9 - 66.8)	7.2	(5.2 - 17.5)	10
hCG - dbc (5 mM dbc)	43.5	(31.7 - 73.8)	8.8	(7.3 - 18.0)	10

Treatment of theca with 1 mM dbc for 6 h did not significantly alter the decline in androgen secretion resulting from 6 h pretreatment with hCG.

Androgen secretion by theca incubated with 5 mM dbc during the 6 - 12 h period was significantly greater ($P < 0.01$[1]) than that secreted by theca incubated with hCG during the 6 - 12 h period. Both groups were pretreated with hCG for 6 h.

1. Wilcoxon matched-pair signed-ranks test. As a matched-pair test was used to determine significance of differences, the results were not pooled if the tissue samples were not 'related'. Hence, the two sets of cont - cont and hCG - hCG results.

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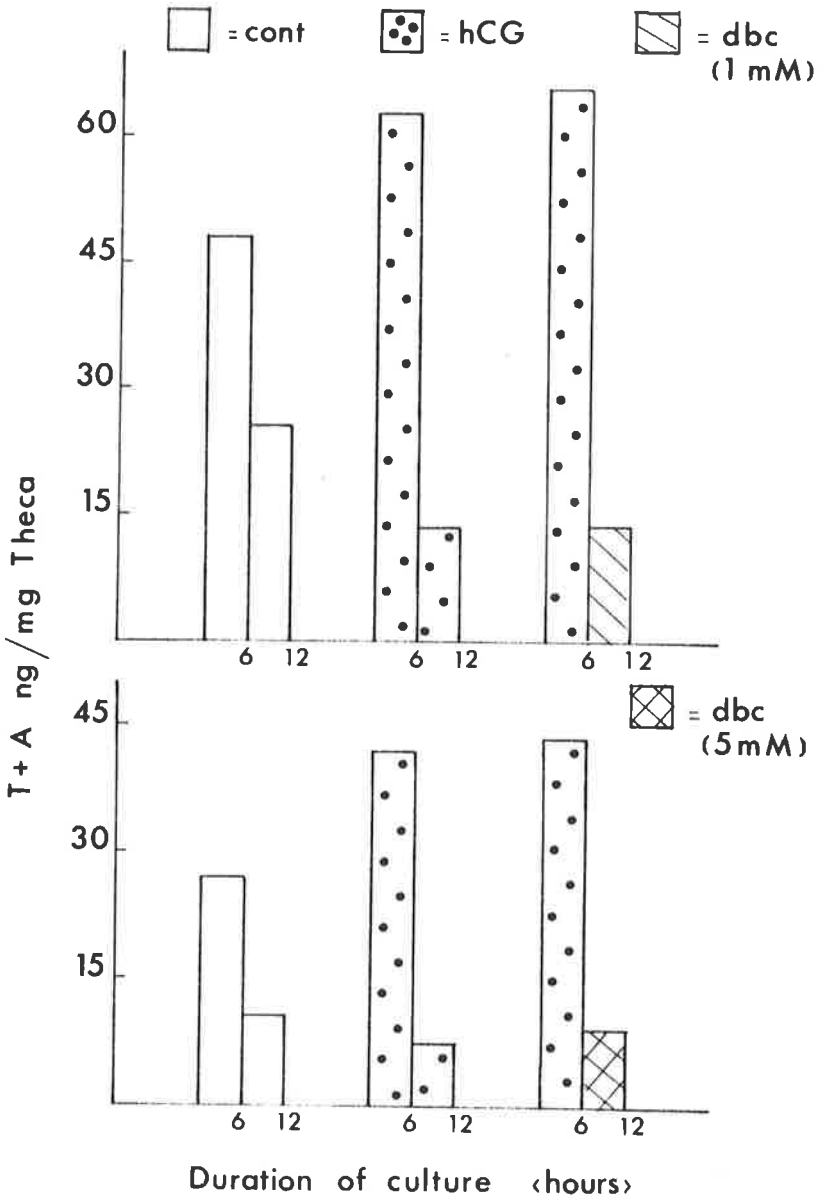

Figure 5.6.1 Effect of dibutyryl cyclic AMP (1 and 5 mM) on the decline in androgen secretion by the theca following 6 h incubation with hCG (5 i.u./ml).

5.7 Discussion

This study demonstrates that both FSH and LH can regulate the responsiveness of their receptor-adenylate cyclase complex in their target cells in the theca and granulosa of the follicle. By virtue of the fact that untreated follicular tissue responds to gonadotrophin treatment, it seems likely that circulating levels of LH and FSH during the luteal phase of the cycle are not sufficient to initiate a desensitization of their receptors. However, the levels of LH and FSH prior to ovulation would appear to be high enough to cause a loss of the cyclic AMP response, both in the follicle and in the newly formed corpus luteum (Hunzicker-Dunn and Birnbaumer, 1976a; Nilsson et al. 1977; and Chapter 7, this thesis).

The desensitization of the hCG induced cyclic AMP response appears to be associated with a change in the steroidogenic responsiveness of the follicle. The inability of hCG to stimulate androgen production by the follicle after an 18 h pre-exposure to hCG may be the result of loss of LH receptors (Conti et al. 1977a) or to inhibition at points subsequent to adenylate cyclase activation (Conti, Harwood, Dufau and Catt, 1977b). The lack of a convincing stimulation of androgen secretion by hCG-pre-treated theca when incubated with dbc supports the proposal that the desensitization of the steroidogenic response of the follicle to gonadotrophins results from inhibition at at least two points, one pre and one post cyclic AMP production. LH has been reported to inhibit the synthesis of cholesterol from acetate in bovine luteal tissue (Armstrong, Lee and Miller, 1971) and of cholesterol from mevalonate in isolated sheep follicles (Douglas, Hamilton and Seamark, 1978), which supports the earlier proposal that the lack of response of isolated rat luteal cells,

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pretreated *in vivo* with LH, to a second incubation with LH, is due to a depletion of progesterone precursor (Armstrong *et al*. 1964). However, rat luteal cells, in a preparation similar to that used in the latter study, also undergo a decline in LH-stimulable adenylate cyclase activity and hCG binding capacity, suggesting again that there may be several sites of inhibition (Conti *et al*. 1976).

There is evidence that at least the initial period of desensitization may be due to a disruption of the coupling between the gonadotrophin receptor and the adenylate cyclase catalytic site. The removal of bound LH from desensitized rat follicles restores hCG binding to control levels but fails to restore the responsiveness of the follicles to LH in terms of cyclic AMP production (Lamprecht, Zor, Salomon, Koch, Ahrén, Lindner, 1977). In contrast, prolonged desensitization persists in the absence of bound hCG or high circulating levels of gonadotrophin and is associated with a reduction in the number of receptors (Conti *et al*. 1976).

While there is evidence that pre-exposure of ovarian tissue to LH results in a loss of responsiveness to further stimulation in the rat (Lamprecht *et al*. 1973) and rabbit (Marsh *et al*. 1973; Hunzicker-Dunn and Birnbaumer, 1976a) there are conflicting reports concerning the role of FSH in these changes in ovarian responsiveness. Hunzicker-Dunn and Birnbaumer (1976a) reported that desensitization of rabbit follicular adenylate cyclase to both LH and FSH was induced specifically by either endogenous LH resulting from mating or exogenous hCG, while exogenous FSH had no effect. Selstam *et al*. (1976) however, observed that pre-incubation of whole rat ovaries with FSH for 120 min prior to a second, 30 min incubation with FSH, resulted in a ten-fold reduction in the cyclic AMP response to FSH compared to a similar group pretreated with LH. Zor

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et al. (1976) have also found that the preincubation of preovulatory rat follicles with FSH results in a loss of response to subsequent stimulation by FSH, while the follicles remain fully responsive to LH. The present study shows that both LH and FSH markedly reduce the responsiveness of theca and granulosa to a second exposure to the same hormone.

We did observe a partial but significant inhibition of the response to the other hormone by both FSH and hCG (table 5.3.1)This is in contrast to a report by Zor et al. (1976) who found little or no desensitization of rat follicles to the heterologous hormone even when, in the case of FSH, a high concentration was used in the preliminary incubation (50 µg/ml NIH-FHS-SIO). Because in the present study a concentration of 1 µg/ml NIH-FSH-S11 was used in the preincubation, it is unlikely that the difference in results is due to LH contamination of the FSH preparation. Secondly, the absence of any significant increase in cyclic AMP levels in granulosa cells from small follicles in response to hCG treatment indicates that the hCG has little, if any, FSH activity, as FSH markedly stimulated cyclic AMP levels in the same preparation (Fig. 5.3.1). The finding that preincubation of granulosa cells with hCG does not reduce the subsequent response of the cells from small follicles to FSH, but does reduce the response of granulosa cells from large follicles suggests that hCG is not acting via binding to the FSH receptor. The reduction of the response of granulosa cells from large follicles may indicate that FSH and LH/hCG receptors exist on the same cell and share a common adenylate cyclase. However, the sharing cannot be complete as in each case the reduction in response to the other hormone was never as great as that to the homologous hormone. In contrast, the reduction of the response of the theca to hCG is difficult to explain as the theca does not respond to FSH, at least in terms of cyclic

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AMP production. It may be that in this preparation, unlike the rat follicle (Zor et al. 1976), the small amount of LH contaminating the FSH is sufficient to cause a desensitization to hCG stimulation. However, while the observed heterologous desensitization may reflect contamination of the gonadotrophin preparations used, or impurities in the cell preparation, the possibility that the inhibition of the response to the other hormone is a specific consequence of the interaction of one gonadotrophin with its receptor cannot be discounted.

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CHAPTER 6

POST PARTUM ANOESTRUS IN THE COW; IS IT THE RESULT OF REDUCED OVARIAN SENSITIVITY TO GONADOTROPHINS?

6.1 INTRODUCTION

The prolonged anovulatory period frequently experienced by the post partum cow significantly hinders the efficient use of the breeding animal in agricultural production. The site and mechanism of the disruption of cyclic ovarian function remains unknown despite considerable research into the problem.

There appears to be little, if any, impairment of pituitary secretion of LH (Scaramuzzi, Radford and Nancarrow, 1977), or of pituitary responsiveness to exogenous gonadotrophin releasing hormone (GnRH), which returns within 6 weeks post partum. Circulating oestradiol - 17 β levels are lower in the post partum cow than in the breeding cow (Scaramuzzi et al. 1977) and may be the result of impaired follicular development. However, an earlier study suggested that post partum anoestrus resulted from the failure of the hypothalamus to stimulate LH release in response to positive feedback action of oestradiol (Radford, Nancarrow and Mattner, 1976). This does not appear to be the case in prolonged post partum anoestrus, as a subsequent study was unable to show a correlation between the time of the first positive response to injected oestrogen and the duration of the post partum anovulatory period (Nancarrow, Radford, Scaramuzzi and Post, 1977).

The elevated levels of prolactin found in the suckling cow (Scaramuzzi et al. 1977) may also be involved in post partum anoestrus, especially in view of the reported regulatory action of prolactin on luteal cell LH receptors (Richards and Williams, 1976) and the inhibitory

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effect of high concentrations of prolactin on progesterone secretion by cultured human granulosa cells (McNatty et al. 1974). If prolactin is involved in post partum anoestrus it is likely to be influencing the ovary since it does not affect the positive oestrogen feedback on LH release (Nancarrow and Radford, 1976).

A possible site for the disruption of ovarian function which had not been investigated was the ovary. In view of the findings of the previous chapter it seemed possible that the sensitivity of the ovarian follicles to gonadotrophins may be impaired either by prolactin or some other mechanism.

6.2 AIM

The aim of this study was to examine the response of theca and granulosa cells, isolated from both post partum and cycling cows, to LH and FSH. Secondly, to ascertain whether prolactin was able to desensitize follicular tissue to LH and FSH. Thirdly, to examine the steroid secretion from follicles isolated from post partum and cycling cows.

6.3 METHOD

In this study 15 lactating and 11 cycling cows were divided into the 5 groups described in Table 6.3.1. As far as possible the cows in the different groups were matched for breed and age. A luteolytic dose of PGF2α analogue (cloprostenol ^{R}, I.C.I., U.K.) and PMSG (1000 i.u., i.m.) was given to some of the cows in an attempt to reduce any effect of circulating progesterone and to increase the yield of follicles. The cows in group V were treated with an inhibitor of prolactin secretion, 2-bromo-α-ergocryptine (CB154, Sandoz).

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Table 6.3.1:

group no.	cow no.	Physiology	Treatment	Days p.p. or day of cycle
I	270	lactating, post partum (p.p.)	food & water removed 36 h before ovariectomy	35
	90			24
	46			
	101			26
	182			24
II	61	cycling, mid luteal	food & water removed 36 h and PG[1] (500 µg) given 24 h before ovariectomy	10
	273			10
	199			15
	281			15
	300			16
III	157	lactating, post partum	food & water removed 36 h, PG and PMSG (1000 i.u. i.m.) given 24 h before ovariectomy	27
	100			23
	173			27
	167			28
	192			29
IV	60	cycling, mid luteal	food & water removed 36 h, PG and PMSG given 24 h before ovariectomy	10
	293			11
	103			15
	272			15
	303			16
	383			14
V	295	lactating, post partum	food & water removed 36 h, CB154[2] given 8 days (100 mg), 6, 4 and 2 days (50 mg) before ovariectomy	30
	99			
	176			32
	155			26
	104			26

1. PGF-2α analogue Cloprostenol [R], I.C.I., U.K.

2. CB154; 2-BROMO-α-ergocryptine (Sandoz).

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Following ovariectomy as many follicles as possible were dissected free from ovarian stroma. The presence and activity of corpora lutea was recorded and, as far as possible, the follicles were measured and counted. The list of follicles given in Table 6.3.2 is not an exhaustive survey of the follicle population of the different ovaries, since loss of follicles during the gross dissection was unavoidable. However, it provides a reasonable estimate of follicle numbers, particularly of those greater than 4 mm in diameter.

Culture Conditions: Once the follicles were dissected from the ovarian stroma they were matched as far as possible with follicles of similar size from the same cow and then one group incubated for 18 h in the presence of prolactin (PRL, NIH-P-S12; 1 µg/ml) and the other in gonadotrophin free medium. The culture conditions were the same as those described earlier (Moor et al. 1973; Chapter 2, this thesis). In some cases more than 1 follicle of similar diameter and from the same cow was incubated in the same culture dish.

After 18 h incubation the medium was removed, frozen and stored at -80°. The theca and granulosa cells were separated by the same method described in Chapter 4. The theca from each follicle was cut in half and placed in 2 tubes/cow; in those groups where the theca was incubated with LH and FSH it was divided between 3 tubes. The granulosa cells from each follicle were suspended in 1.6 ml HEPES-buffered 199 and 0.5 ml placed in 3 tubes/cow. Hormones were added in 10 µl of HEPES over a 5 min period immediately before beginning the 40 min incubation at 37°. The final concentration of LH (NIH-LH-B10) and FSH (NIH-FSH-S10) was 1 and 5 µg/ml of medium, respectively. The incubation was ended

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Table 6.3.2: Approximate size and number of follicles dissected intact from ovaries of cycling and post partum cows.

group no.	cow no.	Corpus Luteum reg.[1]	Corpus Luteum active	Follicles (mm^2) 15	6 - 15	4 - 6	<4	Total
I	270		1		1	8		10
	90[3]	1			3	12		15
	46		1	1	1	17	5	24
	101		1[4]	2		6	6	18
	182	1		1		1	22	24
II	61	1	1	1		20		21
	273	2 - 3	1	2		10		12
	199			1[5]	2	15	4	22
	281		1	1	1	15		17
	300		1	1	2	7	16	26
III	157	2		1	2	2	11	16
	100	1		3			11	15
	173			2	2	4	12	20
	167			1	2	2	12	17
	192			1		10	18	29
IV	60		1	1	4	12		17
	293	1		1		14		15
	103		1	1	3	3	10	17
	272		1	1	2	20		23
	303		1	2	2	13	4	21
	383		1			6	16	22
V	295					5	10	15
	99	1			2	15		17
	176	1				9	12	21
	155			1	2	7	10	20
	104			1	1		25	27

1. Regressed.

2. Classed by diameter, in mm.

3. Only one ovary dissected.

4. First cycle, approx. Day 4.

5. Cystic corpus luteum.

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after 40 min by rapid freezing and the samples stored at -80° until assayed.

Cyclic AMP and steroid assay: Progesterone, oestradiol-17 β, testosterone and androstenedione, and cyclic AMP were estimated in the 18 h incubation medium and in the theca and granulosa samples respectively using the methods described in Chapter 2. Steroid concentration is expressed as pg/ml of medium and cyclic AMP as pmol/mg protein.

6.4 RESULTS

State of Ovary: Twelve of the fifteen post partum cows did not have an active CL in either ovary (Table 6.3.2). The three cows that appeared to have resumed cycling were unfortunately all in group I. Two of the eleven cycling cows did not have an active CL although the ovary of one (cow 199) contained a large, luteinized cystic follicle.

There was no difference in the number of follicles isolated from the cows in each group. Nor was there any obvious difference in the distribution of follicles between the different size classes.

Steroid production i) Effect of prolactin: The steroid production of follicles incubated for 18 h in the presence of prolactin (1 µg/ml) was compared with that of follicles of similar size incubated for 18 h without prolactin. When the steroid production was compared within each group (control versus prolactin, groups I, II, III, IV and V), androstenedione secretion was higher from prolactin treated follicles from group III than from control follicles from group III ($P < 0.005$, Wilcoxon matched-pairs signed-ranks test). However, when all the groups

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are considered together (groups I + II + III + IV + V) there was no significant difference between the control and prolactin treated groups (Fig. 6.4.1).

Steroid production ii) Effect of reproductive state: The control and prolactin data were combined for the between cow group comparison. Comparison of the control and prolactin results separately resulted in the detection of the same differences between groups as the comparison of the steroid production of follicles from control plus prolactin treated groups. Testosterone, androstenedione, and progesterone production during the 18 h incubation did not differ from follicles of cows in each of the 5 groups (Kruskal-Wallis) (Table 6.4.3). Oestrogen secretion however, did differ from follicles from the different groups (Fig. 6.4.2). Follicles from lactating, post partum cows treated with CB154 (V) secreted significantly more oestrogen during the 18 h preincubation period than follicles from either post partum cows treated with PMSG (III) or cycling cows treated with PG (II), however there was no difference between Group V and follicles from the post partum cows of Group I. Secondly, follicles from cycling cows treated with PMSG (IV) secreted significantly more oestrogen than follicles from post partum cows treated with PMSG (III).

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Table 6.4.1: Steroid release by cow follicles after 18 h in culture; control versus prolactin treatment. All cow groups combined. (ng/24 h).

	Steroid	m	95% conf. l.	N
Control	Oestrogen	6.6	(4.5 - 10.5)	52
	Progesterone	3.9	(3.0 - 7.1)	52
	Testosterone	1319.3	(1185.2 - 1527.5)	52
	Androstenedione	419.6	(301.3 - 560.1)	52
Prolactin	Oestrogen	5.5	(4.7 - 9.5)	52
	Progesterone	4.7	(3.1 - 6.1)	52
	Testosterone	1336.4	(1179.9 - 1497.9)	52
	Androstenedione	429.8	(318.5 - 545.1)	52

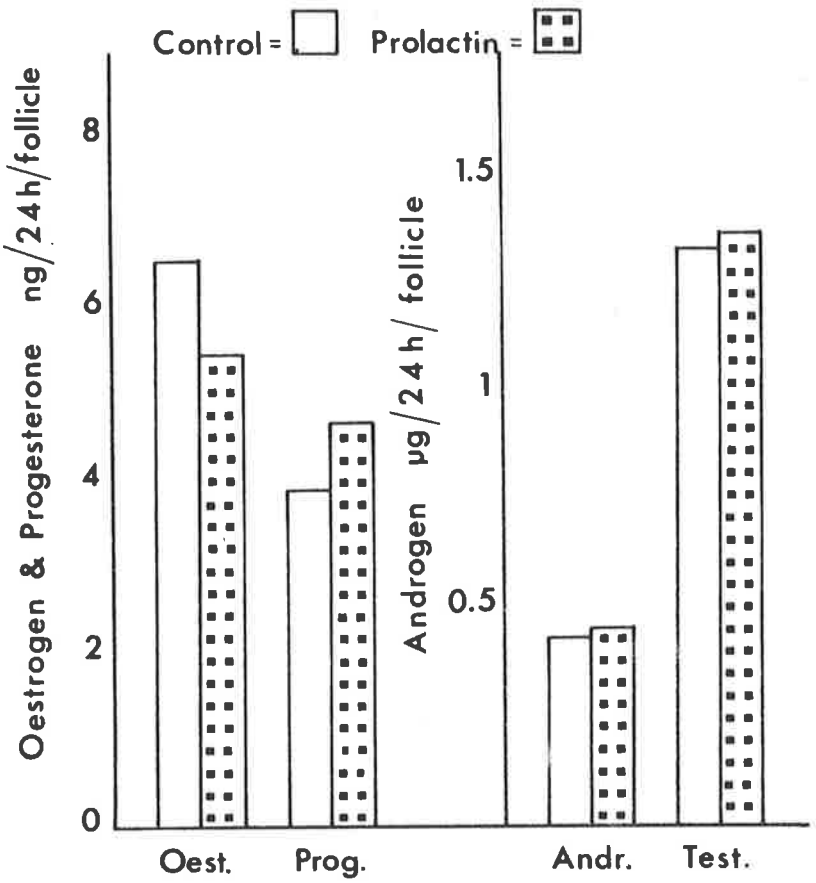

Figure 6.4.1 Steroid release by cow follicles after 18 h in culture. All groups combined; control versus prolactin (1 ug/ml) treatment.

Values plotted are medians. See Table 6.4.1 for further details.

Table 6.4.2: Steroid release by cow follicles after 18 h in culture. Control and prolactin treated groups combined. (ng/24 h).

Group	Steroid	m	95% conf. l.	N
I	Oest.	4.8	(3.3 - 11.3)	20
	Prog.	3.8	(3.1 - 5.5)	20
	Test.	1491.8	(1268.8 - 1697.3)	20
	A'dione	360.5	(255.4 - 492.4)	20
II	Oest.	5.0	(3.7 - 5.7)	20
	Prog.	6.3	(2.7 - 11.4)	20
	Test.	1259.9	(1029 - 1531.7)	20
	A'dione	442.7	(318.5 - 752.5)	20
III	Oest.	4.1	(2.9 - 5.5)	20
	Prog.	4.2	(2.6 - 9.5)	20
	Test.	1073.2	(806.2 - 1344.5)	20
	A'dione	312.0	(216.1 - 473.3)	20
IV	Oest.	11.3	(4.9 - 37.5)	24
	Prog.	3.6	(2.8 - 8.5)	24
	Test.	1482.7	(1257.5 - 1720.2)	24
	A'dione	555.2	(389.2 - 684.8)	24
V	Oest.	9.4	(6.4 - 81.1)	20
	Prog.	4.0	(2.8 - 7.1)	20
	Test.	1271.9	(1114.9 - 1554.9)	20
	A'dione	407.5	(275.7 - 543.1)	20

Oestrogen was the only steroid to be secreted in different amounts by follicles from each of the cow groups.

The oestrogen secretion by the following pairs of cow groups was significantly different at the P = 0.05 level:[1]

II and V

III and IV

III and V

1.= extended Kruskal-Wallis test.

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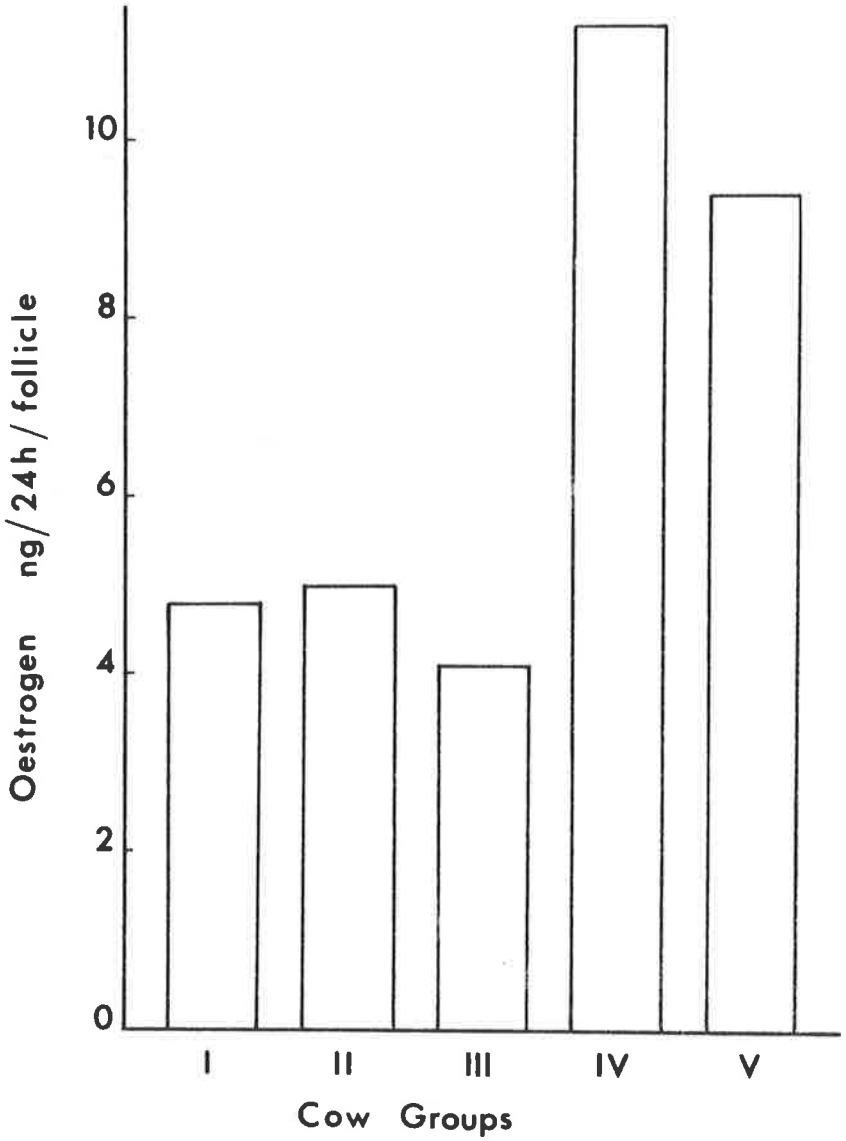

Figure 6.4.2 Oestrogen release by cow follicles from each group after 18 in culture. Control and prolactin groups combined.

Values plotted are medians. See Table 6.4.2 and 6.3.1 for further details.

The following groups are significantly different at the P = 0.05 level:-

II and V
III and IV
III and V

Cyclic AMP production in response to increasing doses of FSH and LH: The result of a limited dose-response experiment are given in the following table.

Table 6.4.3: Cyclic AMP (pmol/mg) production by theca and granulosa after increasing doses of LH and FSH (µg/ml).

	THECA				GRANULOSA						
	LH				LH				FSH		
cow no.	0	0.2	1	5	0	0.2	1	5	0.4	2	10
176	N.D.	2.1	6.9	15.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
167	5.9	18.1	29.8	25.0	N.D.	N.D.	N.D.	N.D.	13.1	25.0	25.3
272	N.D.	6.0	32.9	34.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
46	4.1	7.2	4.3	7.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

The theca and granulosa from one or two follicles from each of the four cows was distributed between 4 and 7 tubes respectively. The theca was incubated for 40 min at 37° in medium containing 0, 0.2, 1 and 5 µg LH per ml. The granulosa cells were incubated in medium containing 0, 0.2, 1 and 5 µg LH per ml and 0.4, 2 and 10 µg FSH per ml.

A concentration of 1 ug LH per ml was sufficient to measurably increase cyclic AMP levels in the theca. In contrast none of the doses of LH increased cyclic AMP levels in the granulosa cells. Each concentration of FSH increased cyclic AMP levels in the granulosa from only one of the cows, while the level in the granulosa of the remaining cows was not detectable.

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Cyclic AMP production i) Effect of prolactin: Prolactin reduced the response to LH of theca of follicles from lactating post partum cows (I and V; $p < 0.01$ and 0.04 respectively, Table 6.4.4). In addition, the response to FSH by the granulosa cells from group I cows was reduced in the follicles pretreated with prolactin. The response of the theca and granulosa from cycling cows to LH or FSH was unimpaired by the pretreatment with prolactin (Table 6.4.5).

Cyclic AMP production ii) Effect of reproductive state: The reproductive state of the cows did not significantly affect the changes in cyclic AMP levels in the theca or granulosa in response to LH or FSH.

Cyclic AMP production iii) Effect of LH and FSH: In each group FSH increased cyclic AMP levels in the granulosa cells. LH significantly increased cyclic AMP levels in the theca but not in the granulosa (Fig. 6.4.3). In addition, a portion of theca from five cows (1 from each group) was treated with FSH (5 µg/ml). The increase, if any, in cyclic AMP levels in the theca of four of the cows was considerably less than in the LH treated group. However, FSH treatment increased cyclic AMP levels over that of LH in the theca of one cow. Whether this response was due to granulosa cell contamination of the theca or to the presence of FSH receptors in the cow theca remains to be determined.

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Table 6.4.4: Effect of prolactin pretreatment on cyclic AMP levels in theca and granulosa following a 40 min incubation with LH (1 µg/ml) or FSH (5 µg/ml). Results are expressed as pmol/mg protein.

	CONTROL			PROLACTIN				
Group I	m	95%[1]	R	m	95%	R	N	P[2]
Theca cont.	0.1	(0 - 2.2)	3.0	0	(0 - 1.5)	3.8	8	N.S.[3]
LH	13.2	(5.1 - 143)	4.5	15.4	(6.4 - 70)	0	8	0.01
Gran. cont	N.D.[4]			N.D.			8	N.S.
LH	N.D.			N.D.			8	N.S.
FSH	7.4	(0 - 230)	4.5	17.0	(0 - 180)	0	8	0.01
Group II								
Theca cont.	0.7	(0 - 6.2)	2.5	0.4	(0 - 4.6)	2.5	10	N.S.
LH	23.6	(1.8 - 64)	5.0	7.9	(1.7 - 52)	6.3	10	N.S.
Gran. cont.	N.D.			N.D.			10	N.S.
LH	N.D.			N.D.			10	N.S.
FSH	4.2	(0 - 197)	5.0	11.9	(0 - 81)	5.0	10	N.S.
Group III								
Theca cont.	1.0	(0 - 4.1)	4.7	1.5	(0 - 3.6)	2.3	8	N.S.
LH	17.1	(4.8 - 54)	3.5	11.8	(3.4 - 193)	7.5	8	N.S.
Gran. cont.	N.D.			N.D.			8	N.S.
LH	N.D.			N.D.			8	N.S.
FSH	47.8	(23.8 - 152)	3.3	26.5	(0 - 151)	5.0	8	N.S.
Group IV								
Theca cont.	0	(0 - 1.9)	3.5	0	(0 - 1.8)	1.5	8	N.S.
LH	7.2	(2.6 - 46)	4.8	13.9	(3.4 - 27)	3.5	8	N.S.
Gran. cont.	N.D.			N.D.			8	N.S.
LH	N.D.			N.D.			8	N.S.
FSH	10.7	(0.2 - 54)	5.0	7.1	(0 - 50)	2.0	8	N.S.
Group V								
Theca cont.	1.3	(0 - 4.9)	3.8	0	(0 - 3.8)	4.5	8	N.S.
LH	17.4	(4.4 - 35)	4.7	11.0	(2.5 - 64)	3.0	8	0.04
Gran. cont.	N.D.			N.D.			8	N.S.
LH	N.D.			N.D.			8	N.S.
FSH	40.0	(0 - 126)	4.5	15.3	(0 - 169)	3.0	8	N.S.

1. 95% confidence limits
2. P obtained by comparing control and prolactin pretreated groups using Wilcoxon matched-pair signed-ranks test.
3. not significant (P > 0.05).
4. not detectable.

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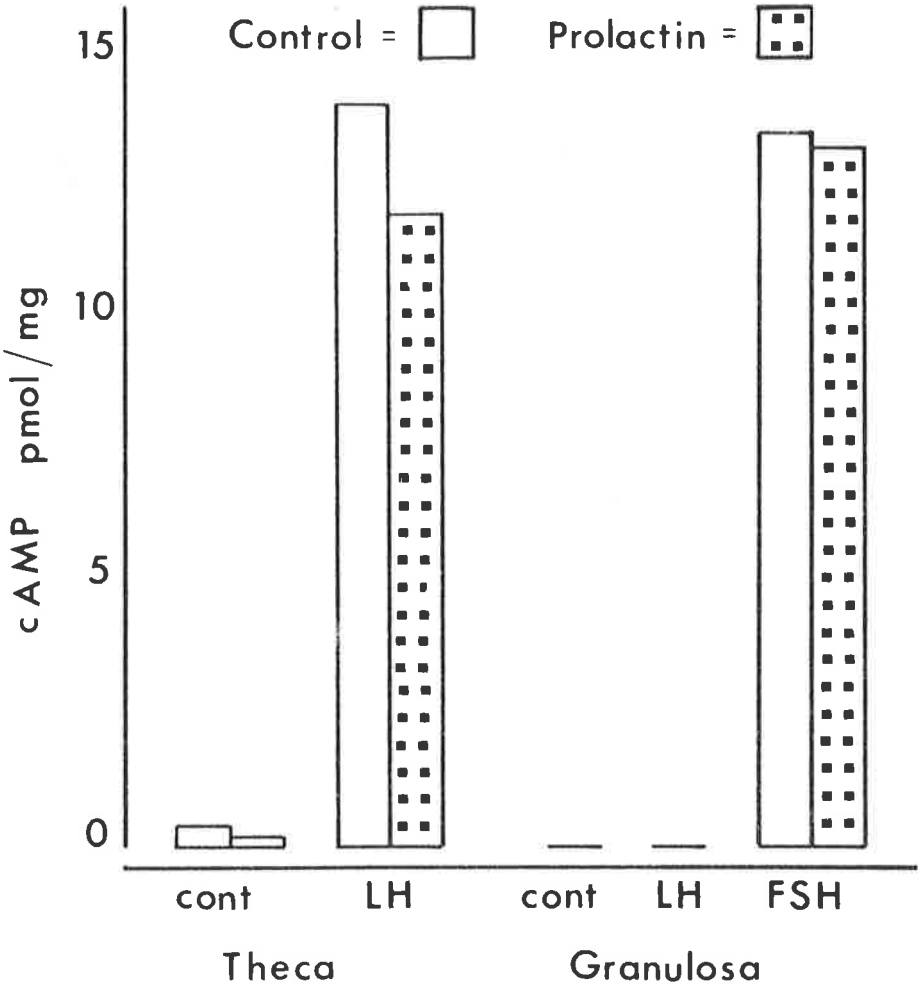

Figure 6.4.3 Changes in cyclic AMP levels of theca and granulosa preparations following 40 min incubation with LH (1 ug/ml) and FSH (5 ug/ml). Comparison of the response of tissue isolated from follicles of control and prolactin (1 ug/ml) pretreated groups. All cow groups combined.

Values plotted are medians. See Table 6.4.4 for the effect of gonadotrophin treatment on tissue isolated from follicles of individual cow groups.

6.5 DISCUSSION

This study has shown that the gonadotrophin responsiveness of follicular adenylate cyclase is not suppressed in the post partum cow. This conclusion is supported by the apparent lack of any impairment of follicular development in the post partum cows. However, it is likely that the follicles examined in this study were not preovulatory follicles since none of them contained granulosa cells responsive to LH. The largest follicle examined was 11 mm in diameter, however it is clear from Table 6.3.2 that a number of the ovaries contained follicles up to and greater than 15 mm. Unfortunately, for practical reasons, these follicles were not examined. It is therefore, still possible that some impairment of the preovulatory follicle could prevent ovulation in the post partum cow.

The differences in oestrogen secretion by follicles from the different groups are not readily explained, however, two things do emerge. Firstly, the oestrogen secretion by follicles from post partum cows(I) was not lower than the secretion by follicles from cycling cows (II). This would appear to be contrary to the findings of Scaramuzzi et al. (1977) who showed that circulating oestrogen levels were lower in the post partum cow than in the cycling cow. This difference is most likely due to the fact that 3 of the 5 cows in group I had recommenced cycling at the time of ovariectomy. In addition, the difference may be a reflection of the follicle population examined in this study since, at least in the sheep, the largest follicle in the ovary is the major source of oestrogen (Moor et al. 1973). As the steroid production by the largest follicles was not examined an impairment of oestrogen secretion by these follicles would have been missed. Interestingly, PMSG treatment

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did not affect the oestrogen secretion by follicles of post partum cows but did increase the secretion by follicles from cycling cows. This suggests that there may be some reduction in the response of follicles to gonadotrophins in post partum cows. The increase in oestrogen secretion of follicles from post partum cows in which prolactin secretion was inhibited (V) supports this proposal. However, once again the presence of cycling cows in Group I makes interpretation difficult as oestrogen secretion of follicles from Group I and V was not significantly different.

The effect of prolactin treatment on cyclic AMP production is also difficult to explain. A possible interpretation of the reduced response to hCG of theca of prolactin treated follicles from post partum cows in Groups I and V may be that the exposure to endogenous prolactin increases the sensitivity of follicles to an inhibitory action of subsequent exposure to prolactin. This effect may be offset by PMSG treatment of cows in Group III.

The lack of effect of prolactin treatment on steroid production may be due to the brevity of the preincubation period. The 18 h incubation with prolactin may not be sufficiently long to alter the responsiveness of the follicles as it takes several days of culture of human granulosa cells with prolactin before progesterone synthesis is inhibited (McNatty et al. 1974).

In summary, this preliminary examination of follicular responsiveness of post partum and cycling cows suggests that there may be some suppression of oestrogen secretion and responsiveness to gonadotrophins in the post partum cow. Prolactin may be involved in these changes, however, further studies are required to fully explore the possibility that post partum anoestrus is the result of an inhibition at the ovarian level.

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CHAPTER 7

EFFECT OF HUMAN CHORIONIC GONADOTROPHIN ON THE RELEASE OF CYCLIC AMP AND STEROIDS FROM THE SHEEP OVARY AT DIFFERENT STAGES OF THE OESTROUS CYCLE

7.1 INTRODUCTION

It is now generally accepted that adenylate cyclase plays a crucial role in the response of ovarian tissues to LH through the production of cyclic AMP which acts as an intra-cellular messenger. There are several reports that LH causes not only a rise in the tissue content of cyclic AMP, but also an increased release of this necleotide from ovarian tissue (Ahrén et al. 1974; Selstam et al. 1976; Chapter 3 this thesis). While it is probable that this release is part of a mechanism through which intracellular levels of cyclic AMP are regulated (Rosberg et al. 1974), it is possible that the nucleotide could also be acting extracellularly as a diffusion activator as suggested in Chapter 3 (this thesis). Apart from studies in the rabbit (Selstam et al. 1976) the magnitude of the cyclic AMP release from the ovarian tissues in vivo is uncertain.

7.2 AIM

The present study was undertaken to determine the effects of hCG on cyclic AMP release by sheep ovaries in situ. Observations were made at different stages of the oestrous cycle in view of the recent finding (Hunzicker-Dunn and Birnbaumer, 1976 a, b) that there were cyclic variations of hormone stimulated cyclic AMP response in the rabbit.

As a further indication of the changes in responsiveness of the corpus luteum during the cycle, progesterone secretion rate was

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measured in seven of the ewes. In addition, oestrogen and testosterone secretion was measured in order to ascertain follicular steroidogenic responsiveness during the cycle.

7.3 METHOD

Animals: Nineteen ewes of mixed breed (mainly Marino crossbreds) were used in this study. The ewes were selected from a flock of cycling sheep held at the University Research Station, Mintaro. Oestrous was detected by running the sheep with a vasectomized ram fitted with a harness and marking crayon (Sironsinc) and observing the ewes daily for the presence or absence of marks. Each ewe had at least two complete oestrous cycles recorded before being brought to the laboratory animal house where they were held for at least one week prior to surgery. There they were fed lucerne chaff and water ad libitum.

Surgery: Anaesthesia was induced with sodium pentobarbitone (Sagatal, May and Baker, Australia) 30 mg/kg i.v. and maintained with a halothane-oxygen mixture (Fluothane, I.C.I., Aust., 1 - 1.5%; 2 l/min O_2). The right femoral or the left branchial artery was cannulated in order to collect arterial samples and for injections. Following a low mid-line laparotomy and heparinization (Heparin Injection B.P., Allen and Hanbury, Aust., 25000 i.u., i.a.) one or both of the ovarian veins were cannulated and connected by means of a loop of siliconized plastic tubing (inner diameter 3 mm) to the left jugular vein. The loop was passed through the abdominal incision and a drip chamber was kept in a fixed position 25 cm from the ovarian vein at the level of the ovary. The connections between the ovarian vessels were ligated in order to prevent blood of uterine origin from entering the ovarian vein. At the completion of the surgery,

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the animal was left undisturbed for 15 min to allow ovarian blood flow to stabilize. Ovarian venous blood was then collected from the drip chamber of the loop for one minute periods with intervals of 2 - 5 min, into pre-weighed ice-chilled tubes which were immediately centrifuged and weighed. Simultaneously, arterial samples were collected and centrifuged. Fifteen minutes after the first blood sampling, 500 i.u. of hCG was given i.a. and a number of ovarian venous and femoral arterial blood samples were then collected up to 60 min after the injection of hCG. Plasma samples were assayed immediately or stored at -20^{o} until analysis.

Cyclic AMP Assay: An aliquot (0.5 ml) of the chilled plasma was added to three volumes of chilled ethanol and centrifuged. The protein-free supernatant was then assayed for cyclic AMP as described in Chapter 2 (this thesis.)

Steroid Assay: Progesterone, oestrogen and testosterone concentrations were determined in the plasma by the radioimmunoassay procedures described in Chapter 2 (this thesis)

The ovarian secretion rates of cyclic AMP and progesterone were calculated from the arterio-venous differences in concentrations of the compounds across the ovary, and from the ovarian venous plasma flow. The haematocrit was determined in each blood sample.

7.4 RESULTS

Where possible, sample medians are given together with 95% confidence limits.

In the conscious ewe, the concentration of cyclic AMP of the

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arterial plasma was 22.9 (12.8 - 41.2) pmol/ml [Median (95% confidence limits)] which is comparable to the levels reported by Jarret et al. (1976). Following the induction of of anaesthesia, peripheral levels of cyclic AMP rose to 93.2 (80.3 - 114.8) pmol/ml. There was no significant difference between the peripheral concentration of cyclic AMP and the concentration found in the ovarian venous plasma prior to hCG injection [98.0 (89.7 - 115.3) pmol/ml].

Secretion of cyclic AMP: Figure 7.4.1 shows the release rates of cyclic AMP from the luteal ovary 10 min prior to, and 20 min after, hCG. The secretion rate of cyclic AMP prior to hCG injection ranged from 0 to 32 pmol/min and appeared to be unrelated to the stage of the cycle. Injection of hCG into the femoral artery resulted in an increase in the rate of cyclic AMP release only from luteal ovaries stages at Days 3 - 18 of the cycle. The increase was apparent in 5 of the sheep at this stage of the cycle as early as 2 min after hCG. Five sheep showed a decline in ovarian cyclic AMP secretion after 60 min, while at that time the levels of cyclic AMP secretion rate of the remaining sheep were unchanged. The complete results from two animals are depicted in Fig. 7.4.2. In these ewes both ovarian veins were cannulated to estimate the contribution of the corpus luteum to the cyclic AMP content in ovarian venous blood after stimulation by hCG. The ewe staged at Day 2 did not show any increase in cyclic AMP secretion from either ovary, whereas the ewe at Day 15 showed a 4-fold increase from the luteal ovary and little or no change from the non-luteal ovary. This result was consistent in the 5 sheep between Days 3 - 18 in which both ovarian veins had been cannulated.

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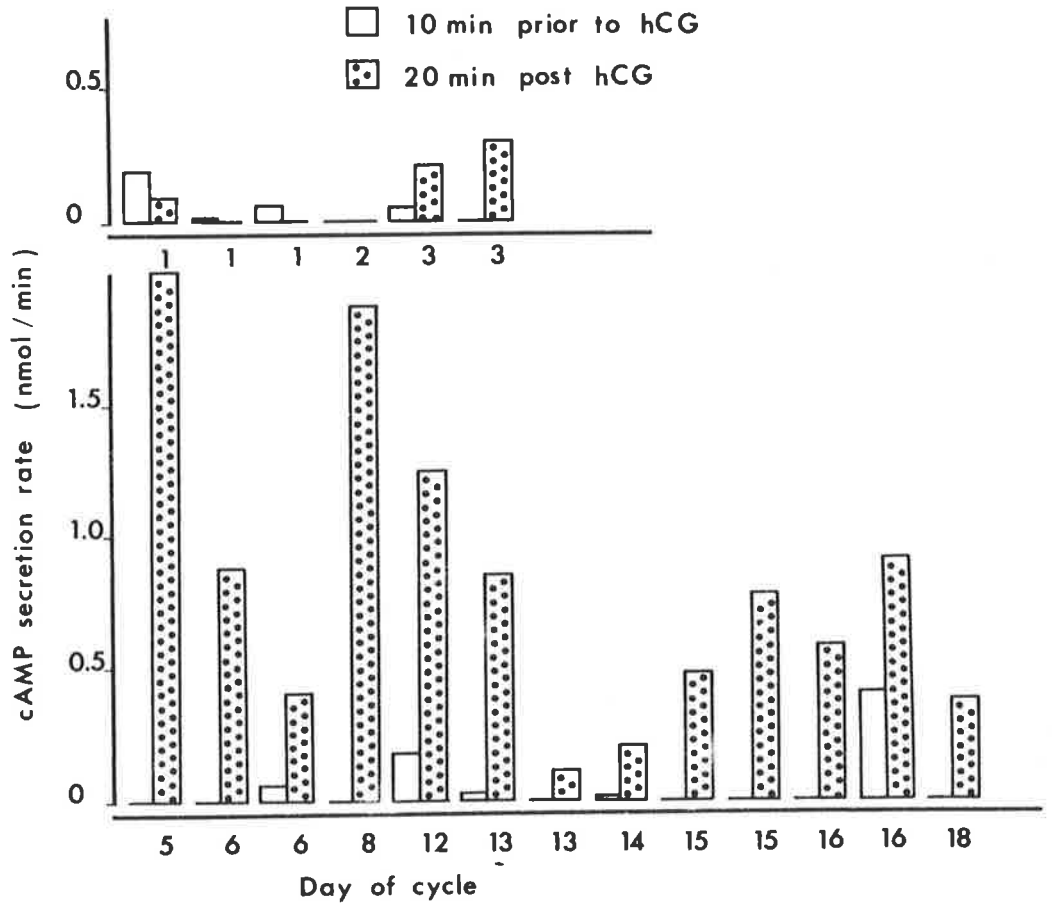

Figure 7.4.1 Cyclic AMP release from the luteal ovary of nineteen anaesthetized ewes at various stages of the oestrous cycle 10 min before and 20 min after an i.a. injection of 500 i.u. of hCG.

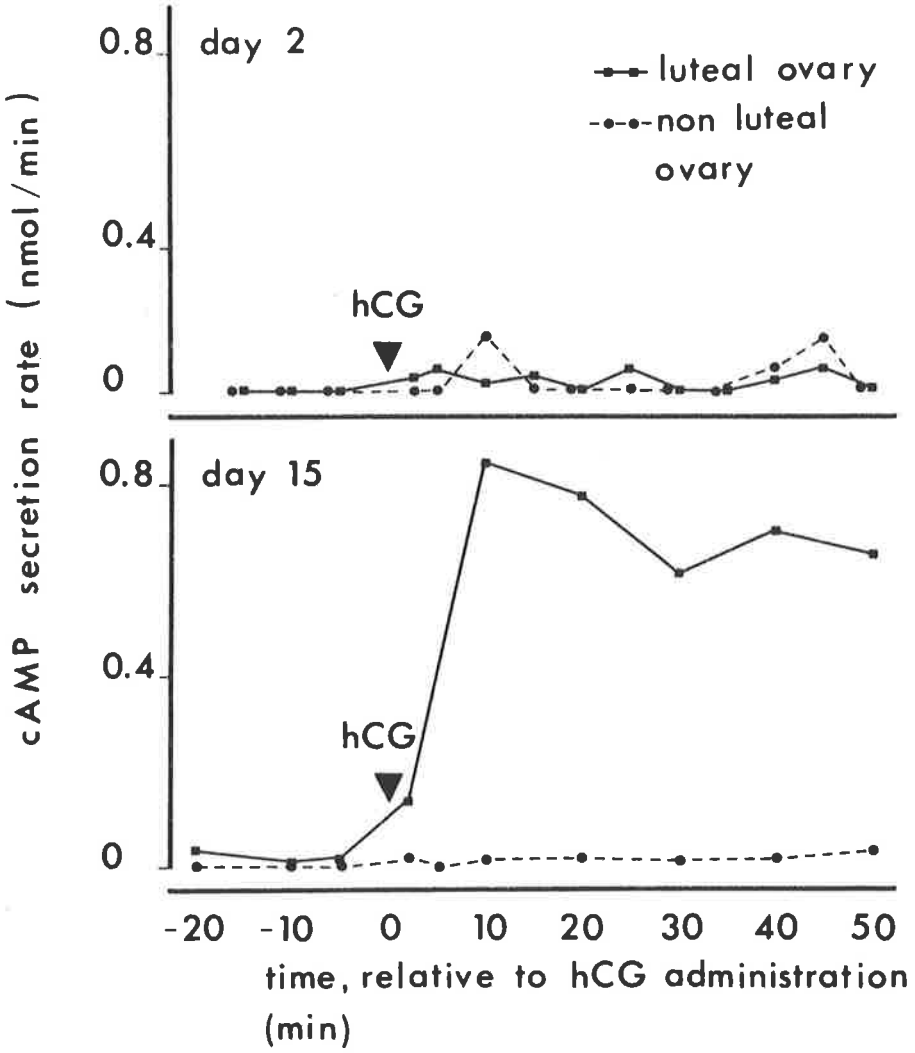

Figure 7.4.2 Cyclic AMP release from the luteal and non-luteal ovary of two ewes at Days 2 and 15 of the cycle. At time 0, an i.a. injection of 500 i.u. hCG was given.

Secretion of progesterone: Table 7.4.1 shows the ovarian secretion rates of progesterone in 7 sheep between Days 1 - 16 of the cycle before and following hCG injection. Two sheep at Days 1 - 2 of the cycle did not respond with an increased progesterone secretion whereas the remaining ewes at later stages of the cycle increased their secretion rates 2 - 4 fold. The progesterone response paralleled the changes in release of cyclic AMP.

The blood flow in the ovarian vein showed no apparent change following hCG injection (Table 7.4.2).

Secretion of oestrogen: Table 7.4.3 shows the ovarian secretion rates of oestrogen in 13 sheep between Days 1 and 18 of the cycle, before and following injection of hCG. Unlike progesterone, oestrogen secretion occurred throughout the cycle. With the exception of 1 sheep at Day 2, all increased oestradiol secretion rate in response to hCG. There did not appear to be any relationship between oestrogen secretion rate and the luteal or non-luteal ovary in those sheep with both ovarian veins cannulated. Regrettably, the ovary with the largest follicle(s) was not identified in this study.

Secretion of testosterone: Table 7.4.4 shows the ovarian secretion of testosterone in 10 ewes between Days 1 and 18 of the cycle, before and following hCG injection. Like oestrogen, testosterone secretion occurred throughout the cycle, was stimulated by hCG injection, and did not appear to be associated more with the luteal or non-luteal ovary.

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Table 7.4.1: Progesterone secretion rate (µg/min) from sheep ovaries before and after an intra-arterial injection of 500 i.u. hCG.

Day of Cycle	Type of Ovary	Cyclic AMP response[1]	Time relative to hCG injection (min) -10	-5	30	40	50
1	luteal	-	0.39	0.57	0.63	0.72	0.60
	non luteal	-	n.d.[2]	n.d.	n.d.	n.d.	n.d.
2	luteal	-	n.d.	n.d.	n.d.	n.d.	0.20
	non luteal	-	n.d.	n.d.	n.d.	n.d.	n.d.
3	luteal	+	5.25	5.11	15.36	not measured	9.56
	non luteal	-	1.05	0.71	0.80	not measured	0.83
3	luteal	+	6.34	8.32	14.61	13.83	13.14
	non luteal	-	0.06	n.d.	0.04	0.01	0.01
15	luteal	+	1.80	1.98	3.45	2.67	3.21
	non luteal	-	n.d.	n.d.	n.d.	n.d.	n.d.
16	luteal	+	2.31	1.81	7.90	3.45	2.98
	non luteal	-	n.d.	0.01	n.d.	n.d.	0.02
16	luteal	+	5.82	14.84	21.28	12.37	11.01
	non luteal	-	0.03	0.06	0.02	n.d.	n.d.

1. = significant rise in the release of cAMP hCG injection

2. = not detectable

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Table 7.4.2: Blood flow in the ovarian vein (ml/min) before and after an intra-arterial injection of 500 i.u. hCG.

Day of Cycle	Type of Ovary	Cyclic AMP and progesterone response[1]	Time relation to hCG injection (min) -10	-5	30	40	50
1	luteal	-	9.6	8.6	5.4	6.0	5.9
	non luteal	-	9.7	9.0	9.6	7.1	7.0
2	luteal	-	4.3	3.4	6.1	6.2	5.6
	non luteal	-	7.6	6.1	7.9	7.6	6.9
3	luteal	+	5.0	6.4	6.1	Not measured	5.7
	non luteal	-	9.7	8.4	6.7	Not measured	6.3
3	luteal	+	12.5	11.2	9.8	9.8	7.7
	non luteal	-	9.3	8.9	7.2	6.3	6.0
15	luteal	+	10.3	8.0	7.7	8.1	8.3
	non luteal	-	4.7	3.3	3.9	3.9	4.3
16	luteal	+	5.8	5.6	3.7	3.3	2.9
	non luteal	-	6.3	6.1	4.4	4.1	3.9
16	luteal	+	13.1	12.7	12.1	9.3	8.6
	non luteal	-	4.3	4.5	2.7	3.6	1.9

1. = significant rise in release of cAMP and progesterone following hCG injection

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Table 7.4.3: Oestrogen secretion rate (ng/min) from sheep ovaries before and after an intra-arterial injection of 500 i.u. hCG.

Day of Cycle	Type of Ovary	Cyclic AMP Response	Time relative to hCG injection (min) -10	-5	30	40	50
1	luteal	-	0.24	0.22	0.82	1.00	1.20
	non luteal	-	0.10	0.14	0.65	0.57	0.61
2	luteal	-	n.d.	n.d.	n.d.	n.d.	0.20
	non luteal	-	0.24	0.11	n.d.	0.23	0.10
3	luteal	+	n.d.	0.10	0.50	0.74	0.51
	non luteal	-	0.70	0.30	1.48	1.58	2.28
4	luteal	+	n.d.	n.d.	2.06	2.17	2.67
	non luteal	-	n.d.	n.d.	1.99	1.80	1.89
5	luteal	+	0.92	0.92	1.05	1.74	1.70
6	luteal	+	n.d.	0.30	1.21	1.17	0.90
8	luteal	+	0.50	0.40	3.48	4.15	4.88
12	luteal	+	0.58	0.45	1.23	0.81	1.07
14	luteal	+	0.15	0.13	0.49	0.34	0.22
15	luteal	+	1.15	0.89	1.79	0.87	2.46
	non luteal	-	0.37	0.10	0.76	0.70	1.00
16	luteal	+	0.51	0.22	1.16	1.03	1.34
17	luteal	+	0.60	0.79	1.85	1.80	1.46
	non luteal	-	n.d.	0.10	0.18	0.22	0.27
18	luteal	+	0.17	0.13	0.29	0.19	0.28
	non luteal	-	0.18	0.20	1.67	1.80	1.78

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Table 7.4.4: Testosterone secretion rate (ng/min) from sheep ovaries before and after an intra-arterial injection of 500 i.u. hCG.

Day of Cycle	Type of Ovary	Cyclic AMP Response	Time relative to hCG injection (min) -10	-5	30	40	50
1	luteal	-	1.34	1.02	1.75	1.50	2.26
	non luteal	-	n.d.	0.19	0.97	1.11	1.06
2	luteal	-	n.d.	n.d.	0.01	0.01	0.01
	non luteal	-	n.d.	0.07	0.06	0.07	0.13
3	luteal	+	n.d.	n.d.	0.26	0.45	0.50
	non luteal	-	0.26	0.62	0.98	1.70	2.87
4	luteal	+	0.17	n.d.	0.32	0.35	0.30
	non luteal	-	0.01	0.03	0.30	0.33	0.71
5	luteal	+	0.38	0.47	0.52	0.66	0.90
8	luteal	+	0.13	0.10	1.02	1.57	1.62
14	luteal	+	0.58	0.33	0.57	0.46	0.74
15	luteal	+	0.50	0.40	1.54	2.31	0.93
	non luteal	-	0.03	0.03	0.66	1.65	0.23
16	luteal	+	0.33	0.31	0.74	1.26	1.31
	non luteal	-	0.10	0.19	0.53	0.80	0.83
18	luteal	+	0.35	0.35	0.43	0.56	0.67
	non luteal	-	0.19	0.03	0.20	0.27	0.25

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7.5 DISCUSSION

The results of this study indicate that gonadotrophic stimulation can elicit a release of cyclic AMP from the sheep ovary *in vivo*, and that the corpus luteum is the main source of the nucleotide released. The finding that hCG elicits no measurable increase in cyclic AMP release from non-luteal ovaries is in contrast to findings in oestrous rabbits in which an injection of LH has been reported to cause a 4-fold increase of cyclic AMP concentration in ovarian venous plasma (Selstam *et al*. 1976). However, the possibility cannot be excluded that the follicles of the ovaries in the present study responded to hCG with a release of cyclic AMP as has been shown *in vitro* in Chapter 3,(this thesis) A follicular release of the nucleotide may have remained undetectable under the *in vivo* conditions of the present study.

The difference in the secretory response of luteal ovaries to hCG found in this study is most likely to be due to a variable responsiveness of the adenylate cyclase system of the corpus luteum. Preincubation of isolated sheep follicles with hCG or FSH results in a reduction in the stimulatory action of a second exposure of the same hormone on cyclic AMP levels in isolated theca and granulosa cells (Chapter 5, this thesis). Similarly, the incubation of preovulatory rat follicles with LH or FSH for 24 h substantially reduced the response of adenylate cyclase to a subsequent stimulation with the homologous hormone (Zor *et al*. 1976). Marsh *et al*. (1973) have previously demonstrated that an injection of hCG *in vivo* resulted in a decline in the ability of rabbit Graafian follicles to synthesize cyclic AMP when incubated *in vitro*. More recently, Nilsson *et al*. (1977) have shown that this desensitization occurs after the endogenous pre-ovulatory surge of

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gonadotrophins in the rat. There is now considerable evidence that the adenylate cyclase in the pre-ovulatory follicle declines in responsiveness as ovulation approaches (Birnbaumer et al. 1976) and remains unresponsive for some time in the newly formed corpus luteum (Hunzicker-Dunn and Birnbaumer, 1976 a, b). The results of the present study indicate that the newly formed corpus luteum in the sheep fails to respond to gonadotrophins both in terms of cyclic AMP and progesterone and that this lack of response persists for at least two days after oestrus. The lack of response of the new corpus luteum may be due to a lack of sufficient luteal tissue to measurable respond to hCG stimulation as well as to a desensitization of follicular adenylate cyclase in the preovulatory follicle as a result of increased LH levels prior to ovulation. The findings are consistent with observations in the sheep that the corpus luteum does not commence progesterone production until 24 - 72 h after the LH peak (Baird et al. 1976 b; Hauger et al. 1977).

The lack of any obvious relationship between oestrogen secretion and the stage of the cycle is in agreement with a study by Baird et al. (1976 b) of steroid secretion by the autotransplanted sheep ovary. They did observe that oestrogen levels were highest 2 - 3 days prior to oestrus, however, the limited number of ewes in the present study preclude a direct comparison. The observation that oestrogen (Table 7.4.3) and testosterone (Table 7.4.4) secretion and responsiveness to hCG stimulation, remains apparently unchanged throughout the cycle, is unexpected in the light of the findings reported in Chapter 5, (this thesis). Since the follicle is the sole source of oestradiol in the sheep (Baird and Scaramuzzi, 1976 c), and the secretion rate and response to hCG remains similar throughout the cycle, it would seem that either the

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desensitization of the follicles to further LH stimulation is short lived (less than 48 h after the preovulatory increase in LH) or it is only the preovulatory follicle that becomes refractory: that is, the follicle that luteinizes and forms the new corpus luteum.. However, an earlier study by Scaramuzzi et al. (1970) revealed a marked fall in oestrogen levels at oestrus, apparently as a result of increased LH levels. It is likely that this was not observed in the present study as the number of sheep examined close to ovulation, in which both ovarian veins were cannulated, was small. Further studies are required to fully characterise the effect of the pre-ovulatory increase in LH on follicular steroidogenesis in vivo in the sheep.

As can be seen in Table 7.4.2, ovarian vein blood flow remained virtually unchanged up to 50 min following the injection of hCG. However, it is well established that LH causes a rapid and significant increase in ovarian blood flow in various species including the sheep (for a review see Bruce and Moor, 1975). The lack of measurable change in ovarian vein blood flow found in this study following hCG is consistent with findings in the rabbit using a similar preparation (Selstam et al. 1976) and may be due to the fact that vessels in the tissues drained by the vein are maximally dilated following manipulation. Another explanation may be that alterations in blood flow in the ovarian vein do not always reflect changes in flow to the ovary proper, since it has recently been shown in this laboratory (Janson, Amato, Weiss, Ralph and Seamark, 1978) that a redistribution of blood flow may take place between the ovary and the tissues along the ovarian vascular predicle as luteal blood flow declines at the time of luteal regression. Thus, the decline in ovarian progesterone

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secretion found in 5 ewes at 50 min after hCG may have been caused by a drop in luteal flow that remained undetectable by measuring the rate of flow in the ovarian vein.

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CHAPTER 8

RELATIONSHIP BETWEEN ATRESIA AND STEROIDOGENESIS IN SMALL FOLLICLES IN SHEEP. DEVELOPMENT OF A PROCEDURE FOR DISCRIMINATING BETWEEN HEALTHY AND EARLY ATRETIC FOLLICLES

8.1 Introduction

The use of cultured sheep follicles in the experiments described in the previous chapters has provided a means of examining a number of aspects of follicular function. However, one difficulty with the method adopted in this study is the often large variation in response of the follicles examined. A possible source of between-follicle variance may result from differences in the state of health of the follicle.

Atresia of antral follicles in sheep takes place at all stages of the ovarian cycle (Brand and de Jong, 1973) with the result that up to 50% of follicles may be atretic (see review by Hay and Moor, 1978). While the later stages of atresia are relatively easy to identify histologically (Hay et al. 1976) this may not be the case in the very early stages. In studies such as the one with which this thesis is concerned it is not practical to routinely examine the tissue histologically. Instead, follicles have been classified on the basis of their gross morphology (Moor et al. 1978) or their oestrogen secretion (Moor et al. 1973). In view of the lack of understanding of the mechanism of atresia, which is largely due to the difficulty of distinguishing between healthy and early atretic follicles, the question arises as to whether follicles assessed to be normal on the basis of their gross morphology (Moor et al. 1978) are in fact from a single population of non-atretic follicles.

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8.2 Aim

The aim of this study was to assess the relationship in small antral follicles, between steroidogenesis and the degree of atresia, as judged from their gross morphological appearance.

8.3 Method

Collection and Classification of Follicles: Ovaries were obtained from the abbatoirs as previously described. Small follicles (1 - 3 mm) were dissected free from ovarian stroma and classified under a stereoscopic microscope into three groups; non-atretic, atretic and unclassified. The criteria on which this classification was based were essentially the same as those reported by Moor et al. (1978). Follicles with a uniform bright translucent appearance, extensive vascularization, and a regular granulosa layer were designated non-atretic. Follicles classified as atretic were dull and poorly vascularized. They often appeared to have holes in the granulosa layer and clumps of material floating in the follicular fluid. Follicles that had a continuous granulosa layer, but had lost the translucent appearance of the non-atretic follicles, were placed in the unclassified group.

The follicles (72 non-atretic, 58 atretic and 45 unclassified) were then cultured for 18 h under the conditions previously described (Moor, 1973) without added gonadotrophin. At the end of the overnight incubation the medium was collected for steroid analysis and several of the follicles from each group were harvested randomly for histological examination. The remaining follicles (42 non-atretic, 33 atretic and 35 unclassified) were cultured for a further 48 h. The medium was collected at the end of each 24 h period and after 48 h the follicles were fixed and examined histologically.

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Steroid Analysis: Oestradiol, testosterone and progesterone were measured in the culture medium by direct radioimmunoassay of 1 - 20 µl of medium (see Chapter 2 this thesis for further details).

Analysis of Data: The differences in steroid output by the non-atretic and atretic follicles was assessed by discriminate analysis using the SPSS sub-programme DISCRIMINANT. The following description of the method is based on the chapter by Klecka on discriminant analysis in the SPSS manual (Nie, Hull, Jenkins, Steinbrenner and Bent, 1975).

The mathematical objective of discriminant analysis is to weigh and linearly combine the discriminating variables in some fashion so that the groups are forced to be as statistically distinct as possible. The discriminating variables are measures of characteristics in which the groups are expected to differ, in this case, steroid production. The aim is to combine mathematically several variables so as to produce, ideally, a single composite measure, at one extreme value of which are clustered non-atretic follicles, and at the other, atretic. Discriminant analysis attempts to do this by forming one or more linear combinations of the discriminating variables.

A statistical measure of the success with which the discriminating variables actually discriminate between groups is obtained from the analysis. In this study, where there were more than two variables, that is oestrogen, testosterone and progesterone on days 1, 2 and 3, it was possible to obtain satisfactory discrimination without including all the variables.

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Classification: Once a set of variables is found which provides satisfactory discrimination for cases with known group membership, a set of classification functions can be derived which will permit the classification of new cases with unknown membership. This serves two purposes; firstly, it enables a check to be made on the reliability of the discrimination, that is, to what extent are new non-atretic and atretic follicles misclassified, and secondly, it enables predictions to be made about the classification of follicles of uncertain morphology.

8.4 Results

Steroid Analysis: The mean (± Standard Deviation) steroid production of the groups of atretic and non-atretic follicles on each of the 3 days in culture is presented in Figure 8.4.1.

In order to ascertain which of the variables contributed most to the separation of the non-atretic and atretic groups, the results were analysed five times, each time with a different set of variables. Progesterone production on Day 1 was undetectable and was therefore excluded from the analyses. Table 8.4.1 lists the percentage of follicles in each group that were correctly classified after examining each set of variables. The success of the classification ranged from 70.8% correct upon considering oestrogen production on Day 1 only, to 78.7% using oestrogen, testosterone and progesterone secretion after 2 days in culture. When all the variables were analysed together, E1* contributed the most information required for separation of the atretic and non-atretic follicles (Table 8.4.2).

* oestrogen production on day 1.

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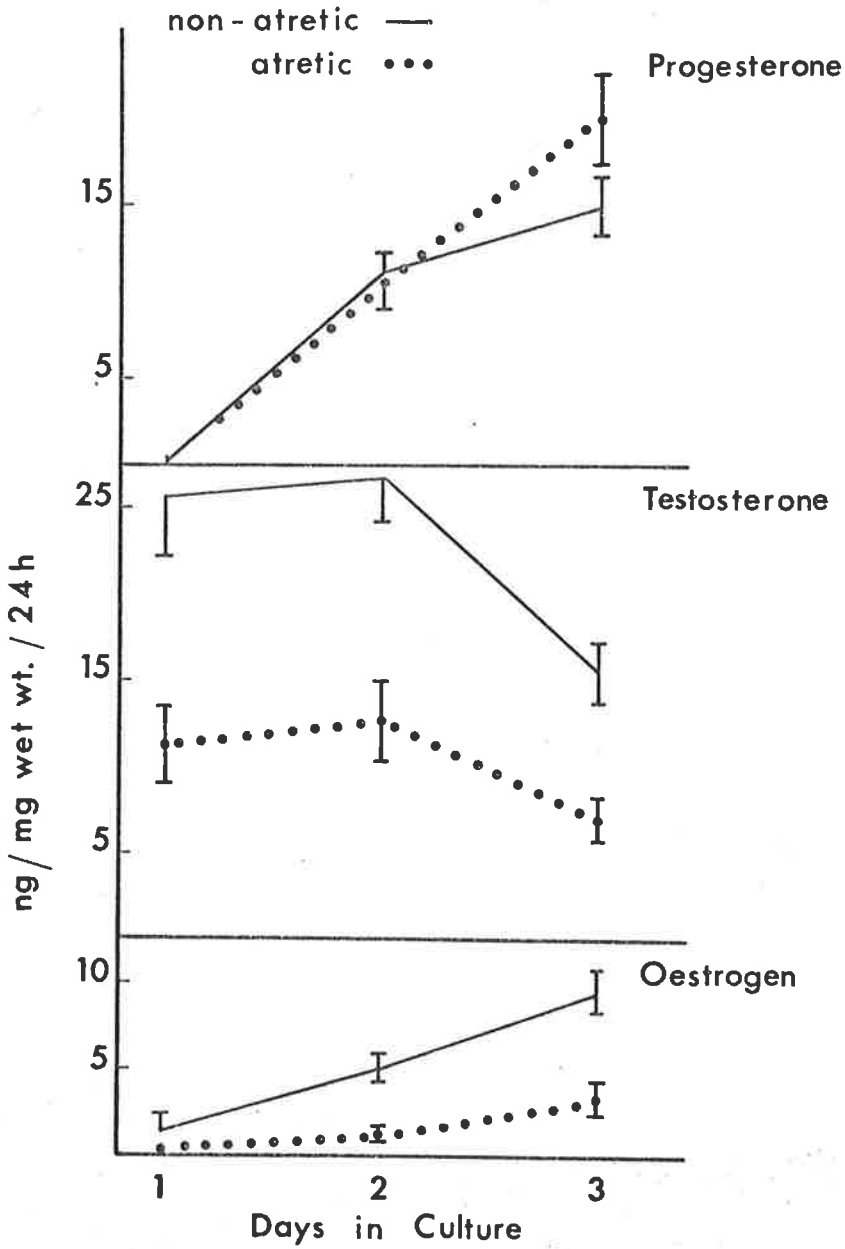

Figure 8.4.1 Oestrogen, testosterone and progesterone secretion of small atretic and non-atretic follicles during 3 days in culture. N = 72 non-atretic and 58 atretic on days 1 and 42 non-atretic and 33 atretic on days 2 and 3. Results are expressed as mean ± Standard Deviation.

Table 8.4.1: Effect of altering the number of variables on the success of the classification of follicles by discriminant analysis.

Variables	Actual group[1]	N	Predicted membership 1	Group 2	Known cases correctly classified
Oest., 1[2], 2, 3 Prog., 2, 3 Test., 1, 2, 3	1 2 3	42 33 35	66.6% 9.1% 40.0%	33.3% 90.9% 60.0%	77.3%
Oest., 3 Test., 3 Prog., 3	1 2 3	42 33 35	69.0% 15.8% 50.0%	31.0% 84.2% 50.0%	76.0%
Oest., 2 Test., 2 Prog., 2	1 2 3	42 33 35	69.0% 9.1% 66.0%	31.0% 90.9% 34.0%	78.7%
Oest., 1 Test., 1	1 2 3	72 58 45	59.7% 13.8% 13.3%	40.3% 86.2% 86.7%	71.5%
Oest., 1	1 2 3	72 58 45	48.6% 1.7% 11.1%	51.4% 98.3% 88.9%	70.8%

1. 1 = non-atretic
   2 = atretic
   3 = unclassified

2. Oestrogen secretion after 18 h, 48 h and 72 h in culture. Similarly for remaining variables.

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Table 8.4.2: Effect of each variable on variance.

Variable entered	F to enter	Wilks lambda	Sig. (1)	Changes in Rao's V	Sig. (2)
Oest., 1	21.11	0.76	0.00	21.11	0.00
Test., 3	5.34	0.70	0.00	7.16	0.01
Prog., 2	1.92	0.68	0.00	2.83	0.09
Test., 2	1.45	0.66	0.00	2.23	0.14
Oest., 3	0.39	0.66	0.00	0.62	0.43
Test., 1	0.17	0.66	0.00	0.28	0.60
Oest., 2	0.07	0.66	0.00	0.17	0.73
Prog., 3	0.02	0.66	0.00	0.34	0.85

The probability values (2) refer to the significance of the change in Rao's V as a result of the addition of each variable to the analysis. They can be interpreted as the significance of the discriminant effect of each variable. Although each variable contributes to the discrimination, oestrogen on Day 1, testosterone on Day 3 and progesterone on Day 2, provide the most information for discriminating between the non-atretic and atretic groups.

The degree of misclassification, by the discriminant analysis, of follicles classified on the basis of their gross morphology as non-atretic and atretic varied slightly depending on which set of variables was included in the analysis. However, the number of non-atretic follicles classified as atretic by the discriminant analysis was always greater than the number of atretic follicles misclassified. Up to 50% of the follicles designated as non-atretic on the basis of their morphological appearance were classified as atretic on the basis of their steroid output, compared to less than 16% of morphologically atretic follicles classified as non-atretic. This difference between the non-atretic and atretic follicles is more clearly seen in Fig. 8.4.2 which is a plot of the discriminant scores for each follicle, obtained by analysis of the

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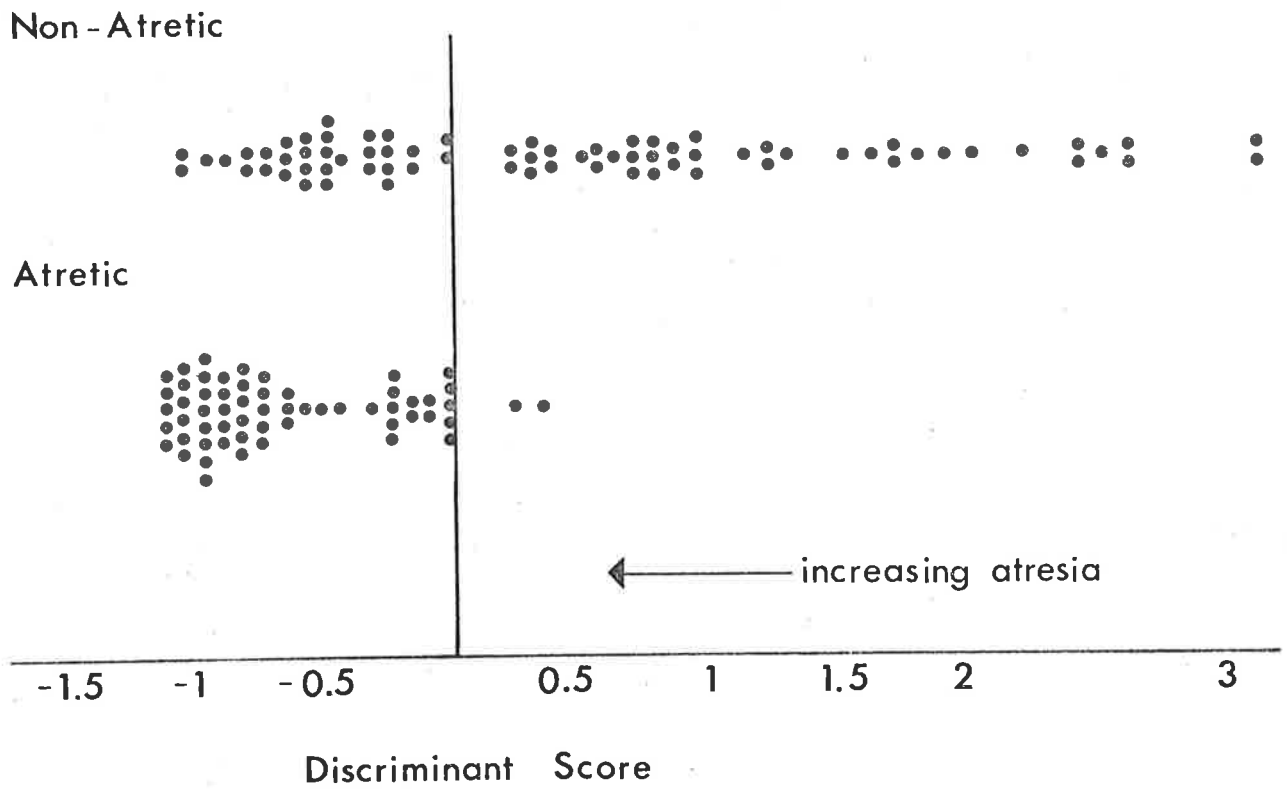

Figure 8.4.2 Plot of discriminant score obtained by analysis of oestrogen and testosterone levels secreted during 18 h in culture by small follicles morphologically classified as atretic and non-atretic.

oestrogen and testosterone secretion into the medium after 1 day in culture. (These two variables were used as the aim was to find the least information required for satisfactory discrimination between atretic and non-atretic follicles). Follicles morphologically classified as atretic were given a negative discriminant score.

Relationship Between Discriminant Score and Histological Appearance: The considerable variation in the behaviour of the non-atretic follicles prompted a comparison of some of the histological features of follicles that fell largely into three groups. They were morphologically non-atretic follicles classified non-atretic, morphologically non-atretic follicles classified as atretic and morphologically atretic follicles classified as atretic.

The occurrence and extent of pyknotic nuclei in the granulosa of follicles in each of the categories is recorded in Table 8.4.3. The area of local pyknosis (Fig. 8.4.3a) was present in all the follicles examined except for 3 which had only been cultured for 1 day. An artefact of the culture system, this was associated with the area of the follicle in contact with the stainless steel grid in the culture dish (Moor et al. 1973). The extent of pyknosis throughout the rest of the granulosa (excluding the localized area) is given under 'scattered pyknosis'. Other indices are listed for some of the follicles; these include the state of development of the cumulus oophorus, the state of the oocyte nucleus and cytoplasm, and the size of the antral cavity. Representative photographs of areas of theca and granulosa from non-atretic follicles and follicles with little and widespread pyknosis are given in Figures 8.4.3a - 5.

There is no doubt that the morphological criteria used for the

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Table 8.4.3: Histological assessment of small follicles classified as atretic and non-atretic by discriminant analysis of their oestrogen and testosterone secretion after 24 h in culture.

Local. pykn.	Scat. pykn.	Cum. ooph.	Oocyte nucleus	Oocyte cytop.	Antrum diam.	Days in culture
a)	Non-atretic - atretic					
+	+	+++	normal	homog.	70 µ	1
none	none	++	res. of meiosis	homog.	35 µ	1
none	none	-	pyknotic	homog.	30 µ	1
+	+	+	-	-	30 µ	1
none	none	+	-	degen.	30 µ	1
+	+	++	res. of meiosis	-	-	3
+	+	-	-	-	-	3
+	++	-	-	-	-	3
+	+	+	-	-	30 µ	3
+	++	++	-	homog.	35 µ	3
b)	Non-atretic - atretic					
+	++	++	-	homog.	50 µ	1
+	++	++	res. of meiosis	homog.	45 µ	1
+	++	++	pyknotic	homog.	75 µ	3
+	+	-	-	-	30 µ	3
+	+	+	-	-	30 µ	3
+	++	-	-	-	-	3
+	++	-	-	-	-	3
+	++	-	-	-	-	3
c)	Atretic - atretic					
+	+++	deg.	normal	homog.	50 µ	1
+	+++	deg.	-	homog.	-	3
+	+++	deg.	-	homog.	-	3
+	++	+	-	degen.	80 µ	1
+	+++	deg.	res. of meiosis	-	-	3
+	+++	++	normal	-	-	3
+	+++	++	normal	-	30 µ	3
+	+++	++	-	-	-	3

Scattered pyknosis: + = 4 pyknotic nuclei per cross section.
++ = widespread pyknosis.
+++ = extensive pyknosis.

Cumulus oophorus: + = very small.
++ = small.
+++ = well developed.

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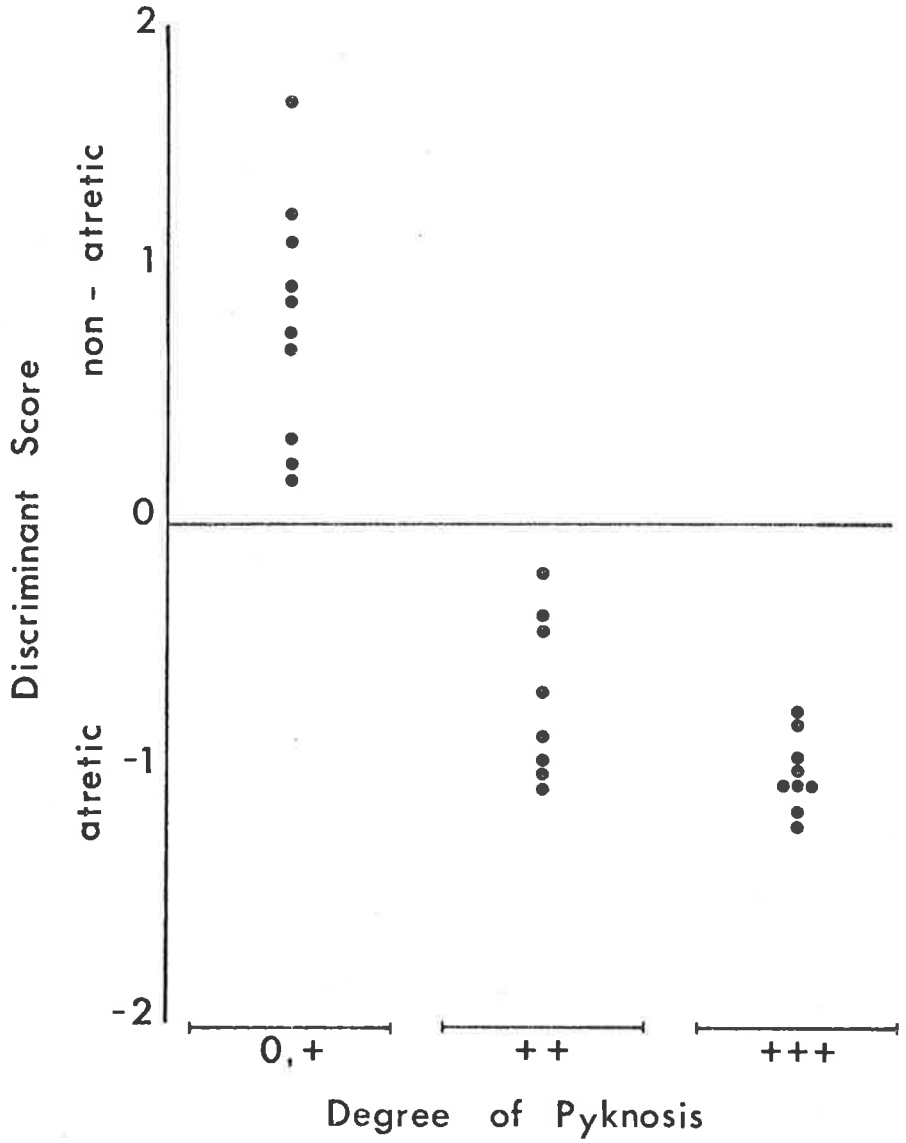

Figure 8.4.3 Plot of discriminant score obtained by analysis of oestrogen and testosterone levels secreted during 18 h in culture by small follicles against the degree of pyknosis of the granulosa examined at the end of the culture period.

initial classification clearly divided the follicles into two groups, one of which was atretic. The atretic follicles contained large numbers of pyknotic nuclei in the granulosa, which occasionally appeared to be breaking away from the theca layer. The cumulus oophorus was sometimes degenerating and often the antral cavity was cluttered with degenerating cells and cellular debris (Fig. 8.4.5a) making measurement of the antral cavity impossible.

The non-atretic follicles contained less pyknotic nuclei than the atretic follicles, and all those examined retained an intact cumulus oophorus. However, there was a larger variation in the degree of pyknosis observed in the non-atretic group than in the atretic one, supporting the proposal that there are a range of possible states between non-atresia and atresia, as indicated by the results of the discriminant analysis (Fig. 8.4.2). When the discriminant score was plotted against the degree of pyknosis (Figure 8.4.3) there appeared to be a relationship between histological atresia as assessed by the degree of pyknosis, and biochemical or steroidogenic atresia as measured by the discriminant score.

While the factor(s) responsible for the initiation of atresia is unknown it has been suspected that an imbalance between androgen and oestrogen production by the follicle may be involved. For this reason an examination was made of the correlations, if any, between oestrogen and testosterone, and oestrogen and progesterone, produced by atretic and non-atretic follicles.

Correlations Between Steroid Production; Relationship with Atresia:

Oestrogen and testosterone production by non-atretic follicles was positively correlated on Days 1 and 2 but not on Day 3 ($P = 0.002$, 0.05 and 0.38 respectively, Table 8.4.4).

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Table 8.4.4: Correlation between steroid production by non-atretic and atretic follicles.

	STEROIDS	CORRELATN COEFFICIENT	P	N
non-atretic	Oe_1vT_1	0.34	0.002	72
atretic	"	-0.03	NS	47
non-atretic	Oe_2vT_2	0.30	0.049	32
atretic	"	0.11	NS	23
non-atretic	Oe_3vT_3	0.06	NS	31
atretic	"	0.24	NS	22
non-atretic	Oe_2vP_2	-0.27	0.067	32
atretic		-0.16	NS	23
non-atretic	Oe_3vP_3	-0.45	0.006	31
atretic		0.14	NS	22

(Oe = oestrogen; T = testosterone; P = progesterone)

In contrast, oestrogen and testosterone secretion by atretic follicles was not significantly correlated on any of the 3 days. Similarly, oestrogen and progesterone production by non-atretic follicles on Days 2 and 3 was negatively correlated (P = 0.07, 0.006 respectively), while the production from atretic follicles was not.

8.5 DISCUSSION

These results show that there is a continuum of physiological states in which the follicle can exist, ranging from non-atresia to atresia. These states, reflected to some extent by the variation in the degree of pyknosis of granulosa cells, are more clearly revealed by an examination of the steroid output of the follicles. The results suggest that there is a correlation between oestrogen output and atresia; that is, atretic follicles secrete less oestrogen than non-atretic follicles

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(also demonstrated recently by Moor et al. 1978). Since there are no discrete groups of non-atretic and atretic follicles the validity is questionable of relying solely on a gross morphological classification in order to select non-atretic or atretic follicles for experimentation, especially as it is clear that the morphologically non-atretic follicles display a range of steroidogenic ability. It appears that a morphological classification of follicles readily separates grossly atretic ones from those that range from being non-atretic to mildly atretic. However, the use of discriminant analysis can provide an objective and reliable method for classifying follicles on the basis of their steroid output. In the analysis done in this study the two groups of follicles were initially classified on the basis of their morphological appearance. Ideally, the groups should be classified on their histological appearance before being used as 'training groups' in the discriminant analysis. This would provide a method of selecting for the degree of atresia desired for a given experimental situation. It is important that the training groups be as precisely defined as possible since, for example, the discriminant classification for the group of small follicles used in this study would not be of any value in an analysis of large follicles as large atretic follicles secrete more oestrogen than small non-atretic follicles (Moor et al. 1978).

Testosterone production by atretic follicles was half that of non-atretic follicles (Fig. 8.4.1) and was not significantly correlated with oestrogen secretion. This is in contrast to non-atretic follicles where oestrogen and testosterone secretion were significantly correlated on Days 1 and 2. The results suggest that atresia is associated both with a decline in testosterone production by the theca and a disruption of the aromatase system.

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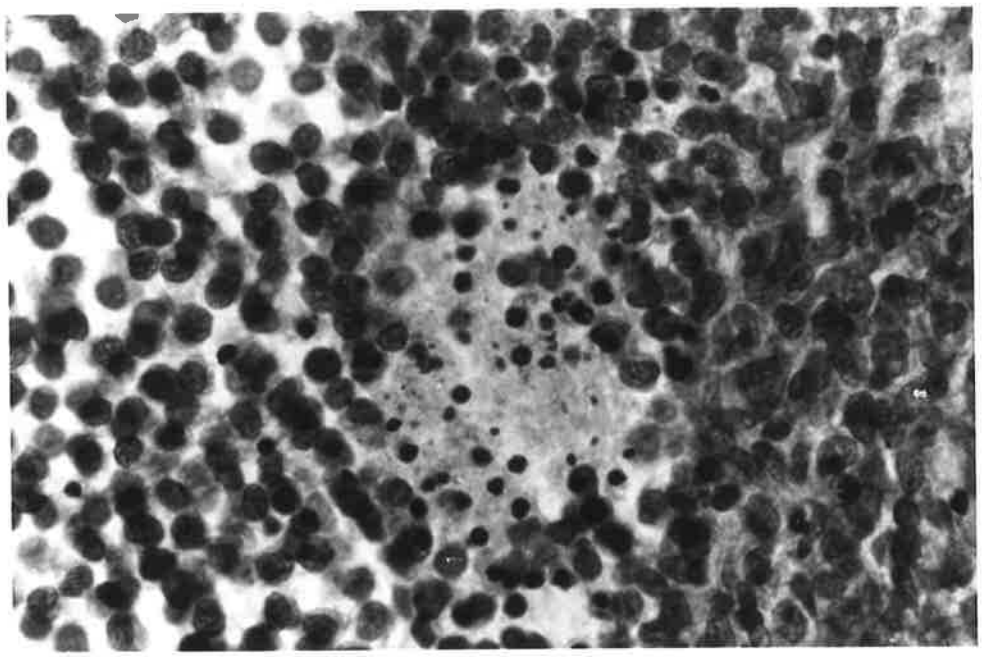
Figure 8.4.3a Non-atretic-non-atretic follicle after 1 day in culture showing area of localised pyknosis.

Figure 8.4.3b Section of the same follicle as above showing a representative area of granulosa away from the localised area of pyknosis.



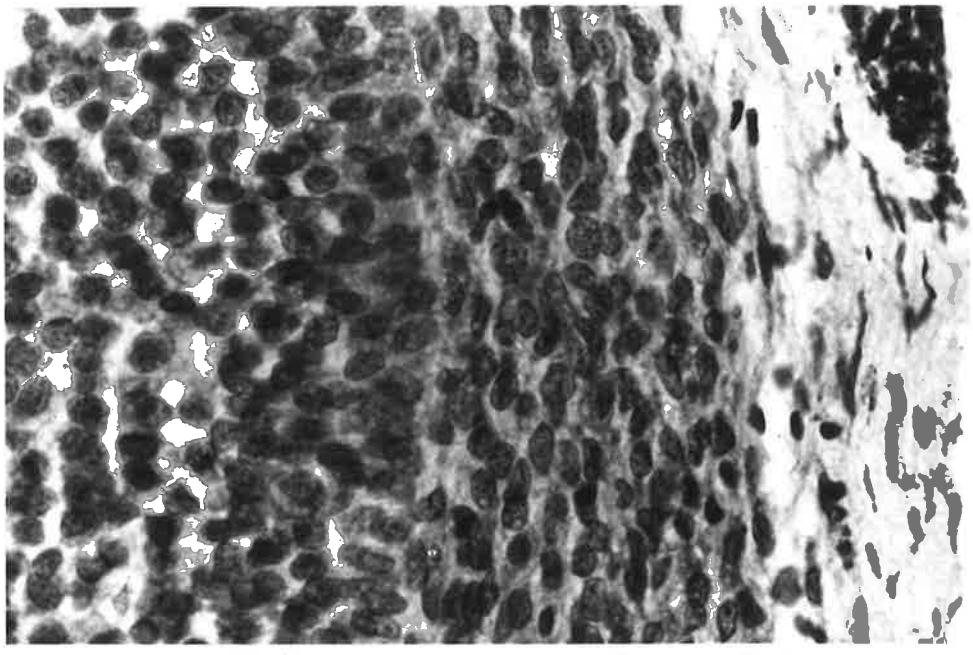
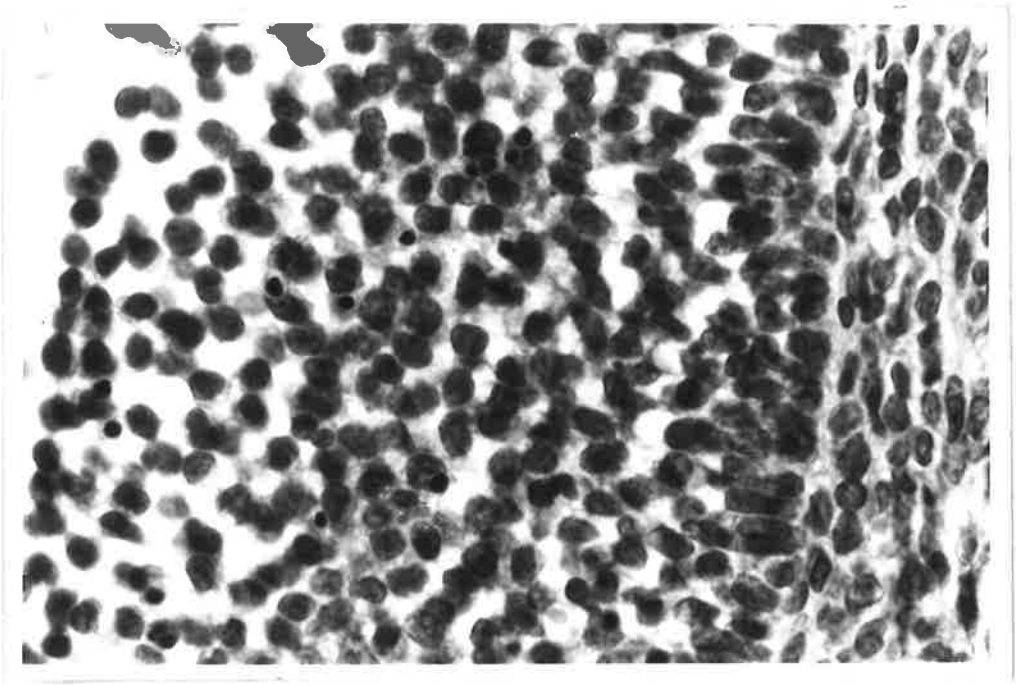


Figure 8.4.4 Areas of granulosa of follicles morphologically classified as normal, but on the basis of their steroid output, classified as atretic. Representative of 'scattered pyknosis'.



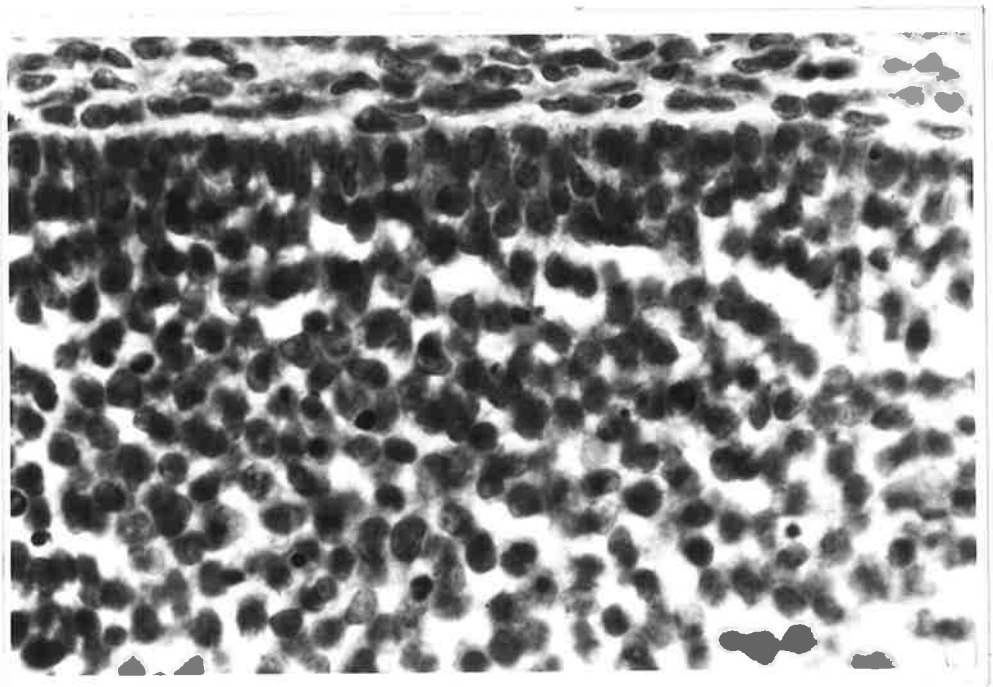
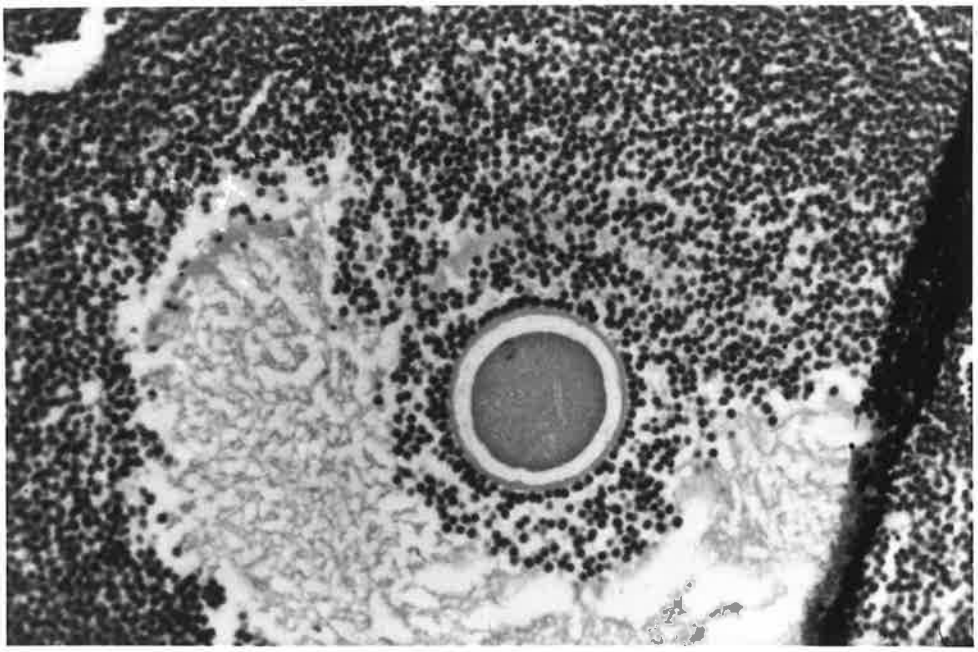
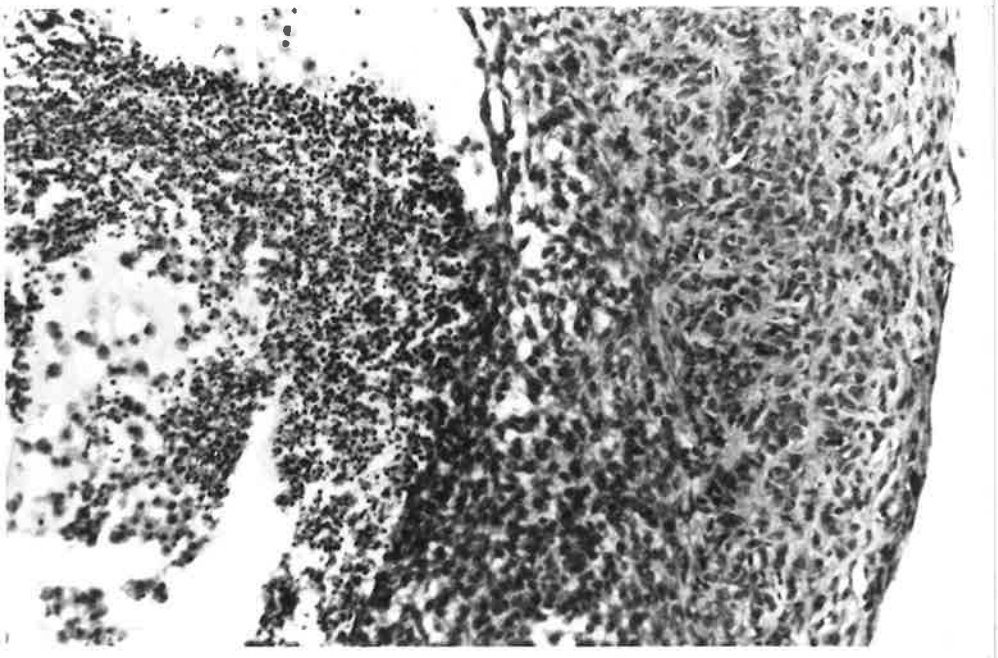


Figure 8.4.5b Atretic-atretic follicle. The section through the oocyte shows a degenerating cumulus oophorus, extensive pyknosis of the granulosa and the oocyte nucleus in telophase, indicating that meiosis has resumed.

Figure 8.4.5a Atretic-atretic follicle. The granulosa extensively pyknotic and degenerating.





CHAPTER 9
GENERAL DISCUSSION

The development and growth of antral ovarian follicles is dependent on the continued influence of gonadotrophins. As a consequence, it is clear that the appearance in follicular tissue of gonadotrophin receptors, and a capacity to respond to gonadotrophic stimulation, is as crucial for successful growth as the presence of gonadotrophins themselves. Until recently, our understanding of the regulatory mechanisms involved in follicular development has been hampered by an inability to examine individual components of the ovary. However, advances in tissue culture techniques have brought with them the opportunity to isolate and examine the properties of relatively homogeneous ovarian cell populations.

The aim of the experiments reported in this thesis has been to examine the effect of gonadotrophins on isolated sheep follicles and follicular tissues *in vitro*, and on intact sheep ovaries *in vivo*, using the production of cyclic AMP and steroids as an index of their response.

While both LH and FSH increased cyclic AMP levels in the tissue of large (4 - 6 mm) intact sheep follicles *in vitro* (Chapter 3), studies in the rat have suggested that the two gonadotrophins may have different sites of action in the follicle (Selstam *et al*. 1976). The separation of the two major cellular components of the follicle, i.e. theca and granulosa, and subsequent incubation with gonadotrophins, revealed that this was indeed the case. In small sheep follicles (1 - 3 mm) the theca contained increased cyclic AMP levels only after incubation with hCG and was not stimulated by FSH. The granulosa, on the other hand, responded only to FSH (Chapter 4). As the steroidogenic capacity of the theca and granulosa is different (Seamark *et al*. 1974; Moor, 1977) the disposition of

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responsiveness to gonadotrophins is reflected in the steroid output of these follicles; at this stage of development (i.e. 1 — 3 mm in diameter) the follicles secrete predominantly androgens, which stem exclusively from the theca (see below), with little or no oestrogen secretion.

The growth of follicles from 1 — 3 mm to 4 — 6 mm in diameter is associated with changes both in steroidogenic output and gonadotrophin responsiveness. The most marked changes occur in the granulosa, which undergoes rapid proliferation (Turnbull et al. 1977) and becomes responsive to hCG, as assessed by cyclic AMP production (Chapter 4). In contrast to the small follicles, the larger follicles secrete significant amounts of oestrogen as well as some androgen (Moor et al. 1978).

It is becoming increasingly clear that follicular development is the result of a co-operative interaction between the theca and granulosa under the influence of pituitary secretions. Neither the production of oestrogen (Moor, 1977), nor the appearance of LH receptors in the granulosa (Nimrod et al. 1976), will occur in isolated granulosa cells, indicating the importance of contact between the granulosa and the rest of the ovary. The requirement for theca androgen production in the development of the granulosa has been suggested by a number of studies, particularly in the rat. The sheep theca (Moor, 1977), like that of the rat (Fortune and Armstrong, 1977) and hamster (Makris and Ryan, 1975, 1977), is the major site of follicular androgen production. Although the best documented role of thecal androgen is as substrate for oestrogen synthesis, androgens may independently influence granulosa steroidogenesis as, at least in the rat, both testosterone and androstenedione have been shown to act synergistically with FSH in stimulating progesterone production by

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granulosa cells (Nimrod and Lindner, 1976; Armstrong and Dorrington, 1976 and Luckey et al. 1977). Evidence from the present study suggested that the granulosa supplies substrate for thecal androgen production (Chapter 5), either as progesterone or pregnenolone (both of which are produced by the sheep granulosa, Seamark et al. 1974). Thecal androgen may therefore be acting as a positive 'feed-forward' regulator of its own synthesis in the theca, and the synthesis of oestrogen in the theca or granulosa, by increasing the availability of C_{21} steroid substrate from the granulosa. Steroid production by the theca and granulosa therefore appears to be regulated by both intra follicular levels of steroids, and by gonadotrophins.

The stage of development of follicles is crucial in determining their response to preovulatory increases in gonadotrophins. The present findings (Chapter 4) that granulosa cells from large, but not small sheep follicles, increase cyclic AMP levels in response to hCG provides an explanation for the observation of Turnbull et al. (1977) that in ovaries of ewes injected with PMSG followed 24 h later by hCG, the majority of follicles less than 3.5 mm in diameter were undergoing early atresia, while those larger were beginning to luteinize. It is likely that hCG stimulates thecal androgen production in small follicles and, in the absence of a capacity to luteinize, the elevated androgen levels result in the atretic changes observed. The larger follicles, while they will not all ovulate, do possess LH responsive granulosa cells and as a result begin to luteinize following exposure to hCG.

The mechanism by which granulosa cells develop a responsiveness to LH is unknown. However, the recent finding that hCG pretreatment can potentiate the effect of FSH on LH and FSH receptor content of rat granulosa

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cells (Ireland and Richards, 1978) suggests the possibility that a substance of thecal origin may be involved, as the theca is responsive to hCG before the granulosa is. In Chapter 3, intact sheep follicles treated with hCG were found to release considerable amounts of cyclic AMP, suggesting that cyclic AMP of thecal origin could influence the development of the granulosa. Cyclic AMP is thought to act as an intra-cell cycle regulator (for a review see Whitfield et al. 1976), but it remains to be seen whether or not changes in extra cellular cyclic AMP levels, which result from LH stimulation of thecal adenylate cyclase, influence granulosa cell development.

In sheep, the oestrogen output from the ovary containing the largest follicle increases gradually and reaches a maximum rate 40 — 48 h before ovulation. This is maintained until approximately 20 h before ovulation and then declines sharply to low levels (Scaramuzzi, Caldwell and Moor, 1970; Moor, Hay and Seamark, 1975). The decline in oestrogen secretion prior to ovulation may be due to a desensitization of follicular adenylate cyclase caused by increasing levels of LH (Chapter 5). This desensitization may result from a loss of gonadotrophin receptors (Rao et al. 1977; Ireland and Richards, 1978). However, the rapid decline in androgen production by sheep theca preparations treated with hCG and a similarly rapid inhibition of rat ovarian aromatase activity by LH (Katz and Armstrong, 1976) suggests that the initial inhibition may be due to receptor occupancy or uncoupling of the receptor-adenylate cyclase complex (Lamprecht et al. 1977). Further, there is evidence that the loss of biological response by different ovarian preparations results from inhibition at several points (Chapter 5 and Conti et al. 1977) and, in the case of oestrogen production, may be due to a specific inhibition of

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aromatase or proteolysis of the aromatase enzymes as suggested by Katz and Armstrong (1976). As ovulation occurs about 24 h after the LH peak in sheep (Hauger et al. 1977), the ovulatory follicle is therefore likely to be unresponsive to further LH stimulation during the period immediately prior to, and following ovulation (Chapter 7). The physiological significance of this period is unknown, although it may be necessary to allow processes normally inhibited by cyclic AMP to proceed. A loss of responsiveness may also be involved in luteolysis. A decrease in the number of hCG receptors in corpora lutea occurs during luteolysis in the rat (Lee, Tateishi, Ryan and Jiang, 1975), and in pig corpora lutea a decline in adenylate cyclase activity has been reported towards the end of the cycle (Anderson, Schwartz and Ulberg, 1974). Similarly, it has been proposed that the lack of response of human corpora lutea of pregnancy, is due to a desensitization of gonadotrophin receptors (Marsh, Savard and LeMaire, 1976), perhaps as a result of the elevated levels of hCG present during pregnancy.

The hormone specific regulation of the response of tissue to hormonal stimulation plays an important part in the development of ovarian follicles. The impairment or lack of hormone receptors is associated with a number of endocrine disorders (see review by Catt and Dufau, 1977) and may be involved in seasonal or post partum anoestrous (Chapter 6). A better understanding of the mechanisms involved in the regulation of hormone responsiveness may provide insight into causes of infertility, or suggest more specific methods for the artificial regulation of fertility.

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