STUDIES ON THE MECHANISM OF ACTION OF GONADOTROPHINS ON STEROIDOGENESIS IN THE OVARY OF THE IMMATURE RAT

by

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DEDICATED TO THE MEMORY OF A

DEVOTED FATHER, CHIEF JAMES OTON EBONG

1905 - 1975

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ABBREVIATIONS

Act.D	actinomycin D
Butyl PBD	(2-4' tert butyl phenyl-5-4' biphenyl)-1,3,4-oxadiazole
c'AMP	cyclic adenosine 3-5- monophosphate
ATP	adenosine triphosphate
^{o}C	degrees centigrade
^{14}C	radioisotope of carbon
Ci	curies (1 Ci = 2.22×10^{12} dpm)
d.b. c'AMP	N^6-2'-O-dibutyrl-adenosine-3'-5-monophosphate
dpm	radioactive disintegrations/min
ED_{50}	dose of drug causing 50% maximum response
FSH	Follicle Stimulation Hormone
g	grams weight
H, h	Hour
HCG	Human chorionic gonadotrophin
^{3}H	tritium
i.p.	intraperiteneally
i.u.	international unit
KRB	Krebs' Ringer Bicarbonate
l	litre
LH	Luteinizing Hormone
M	Molar
min	minute
ml	millilitre
mg	milligram
μ	(prefix) - micro - $\times 10^{-6}$
mRNA	messenger RNA
n	number in a group
NIAMDD	National Institute of Arthritis, Metabolism and Digestive Diseases
NS	not significant
^{32}p	radioisotope phosphorus
ρ	(prefix) - pico - $\times 10^{-12}$
p.d.	per day
PMSG	Pregnant mares' serum gonadotrophin
POPOP	bis phenyl oxazoly benzene
PPO	diphenyl oxazole
RNA	ribonucleic acid
r.p.m.	revolutions per minute

S.C.	Subcutaneous
SEM	Standard error from the mean
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

PHYSIOLOGY AND PHARMACOLOGY

Doctor of Philosophy

STUDIES ON THE MECHANISM OF ACTION OF GONADOTROPHINS ON STEROIDOGENESIS IN THE OVARY OF THE IMMATURE RAT

by Patrick Ekong Ebong

Experiments were designed to examine the effects of treating immature rats with pregnant mares' serum gonadotrophin (PMSG) on several important parameters of ovarian development. PMSG caused an increase in ovarian steroid secretion both *in vivo* and *in vitro*. Plasma oestradiol levels in the PMSG-primed rats reached a maximum in the animals injected 48 h previously (analogues to pro-oestrus) and declined to a minimum in the animals injected with PMSG 72 h previously. Testosterone levels, like those of oestradiol reached maximum in the animals injected with PMSG 48 h previously but unlike oestradiol did not decline abruptly 24 h later. Progesterone levels were maximal in the animals injected with PMSG 72 h previously (i.e. after the expected LH/FSH surge and after ovulation had occurred, as indicated by the presence of ova in the oviducts of the animals).

Human Choronic Gonadotrophin (HCG) added to incubates of the ovaries of animals injected with PMSG 48 h previously caused a marked increase in oestradiol and testosterone secretion whereas the post ovulatory ovaries secreted predominantly progesterone. The oestradiol secreted under these conditions might have been principally due to an enhanced synthesis of testosterone and the production of increased amounts of substrate for aromatization. Evidence is produced to support this hypothesis. The presence of cycloheximide suppressed the HCG-induced steroid secretion, from which it was inferred that protein synthesis is required for the HCG-induced steroidogenesis.

In addition, experiments were carried out to investigate the effect of 5α-reduced androgens on oestradiol secretion from granulosa cells isolated from pre-ovulatory follicles of PMSG-primed immature rats. Our results demonstrate that the major 5α-reduced androgens found in the ovary of immature rat do suppress the aromatization of exogenous testosterone. These results are discussed in the context of the control of follicular development and of the onset of puberty in the immature rat.

CHAPTER 1

INTRODUCTION: A REVIEW OF THE RELEVANT LITERATURE

1.1 INTRODUCTION

Few areas in reproductive biology have received so much attention in recent years as the regulation of ovarian development and function. The reason for this is that within the complex interactions which result in the selection of a few oocytes from the thousands which are present within the ovary lie many potential sites which may be suitable as targets for regulating ovulation. This is obviously important both for controlling the human population and, in animal husbandry, for increasing the reproductive capacity of domestic animals.

The problems associated with the control of follicular development are numerous. They include morphological differentiation of the thecal cells, granulosa cells and oocyte; oocyte/follicle interdependence; the exogenous and endogenous regulation of receptors for protein and steroid hormones; the eventual death of follicles through atresia or their 'rescue' through ovulation and luteinization. The problems are complex but the solutions will provide a deeper understanding of some of the mechanisms involved in ovarian follicular development and function.

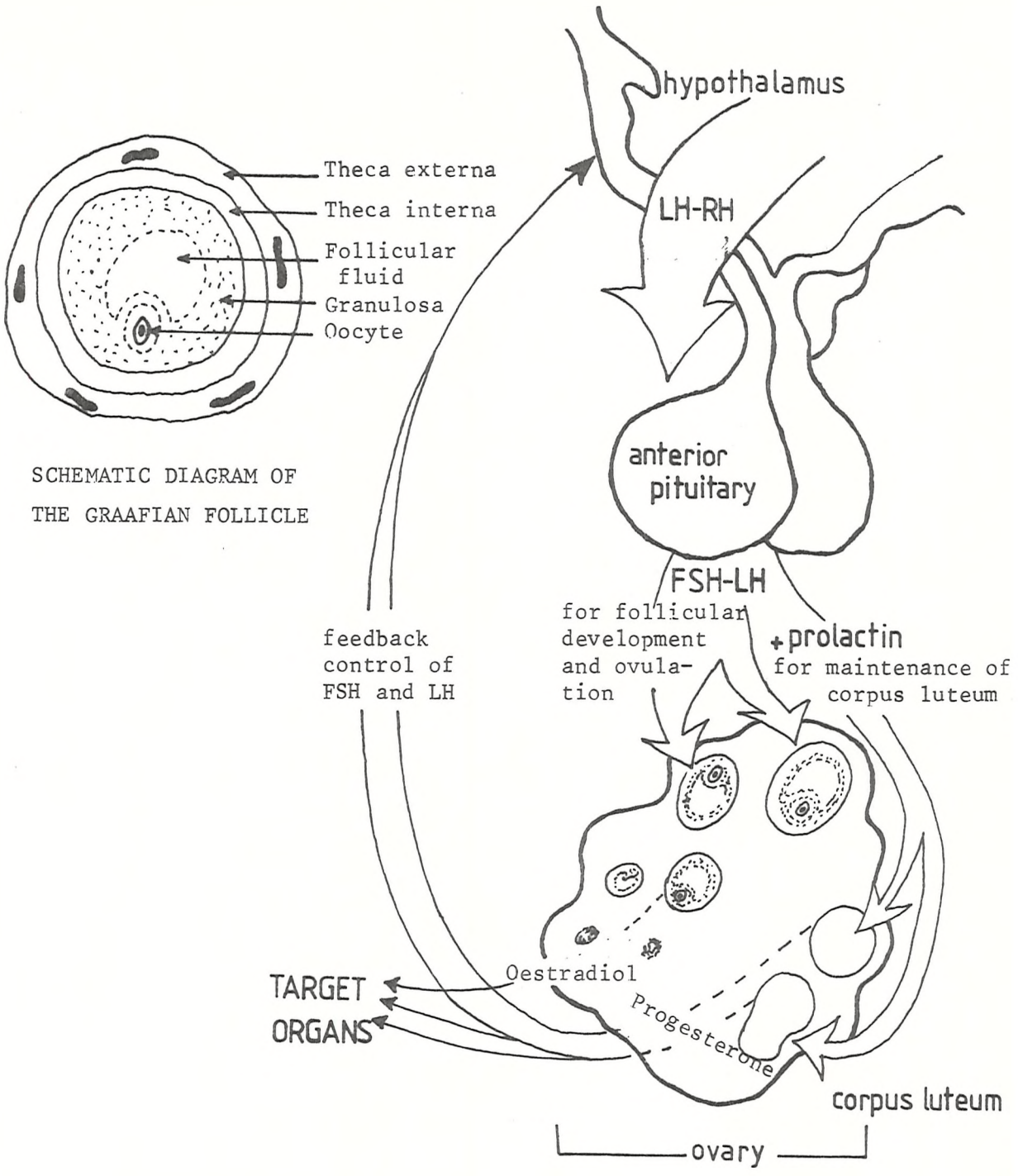
The purpose of this chapter is to review some of the recent studies on the regulation of ovarian follicular development. This review will include some of the literature concerned with: (a) the morphological development of ovarian follicles and their oocytes; (b) the mechanism(s) of action of gonadotrophins, (Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)) and oestradiol within the rat ovarian follicles;

(c) the dependence of follicular development upon gonadotrophins; (d) the role of androgens within the ovary and the regulation of androgen synthesis by LH; (e) changes in steroidogenesis which occur around the time of ovulation; and (f) the role of follicular atresia during follicular development. Although an exhaustive review of the literature is not feasible, I hope this review will bring together several facets of follicular development which are relevant to the subsequent experimental work in this thesis. I also hope to place my work firmly within the scope of current knowledge, so that an overall integrated appreciation of follicular development in the ovary of the rat can be made.

1.2 MORPHOLOGICAL DEVELOPMENT OF OVARIAN FOLLICLES AND THE DEPENDENCE OF DEVELOPING FOLLICLES ON GONADOTROPHINS

The interaction between primordial follicles and growing follicles represents perhaps the most important function of the mammalian ovary. It is this event which ensures that the oocytes are released at a steady rate throughout the reproductive life of the animal, and hence ensures a steady sequence of ovulations. The success of these integrated events depends upon the interplay of several control mechanisms, which lie partly outside the ovary and partly within the ovary itself. (Fig. 1.1). Follicular development has many facets, two of which are: follicular organization, which results in the formation of a pool of non-proliferating small follicles soon after birth; follicular growth, which starts when follicles emerge from the pool of non-growing follicles, during postnatal development or the oestrous cycle. Integrated follicular growth ensures that between the time when the follicle starts

FIGURE 1.1 INTERRELATIONSHIP BETWEEN HYPOTHALAMUS, PITUITARY AND OVARY (ADAPTED FROM SCHULSTER, BURSTEIN AND COOKE, 1976).



The hypothalamus secretes the gonadotrophic releasing hormone (LH-RH) which stimulates the secretion of both Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) from the anterior pituitary gland. Follicular growth is stimulated by FSH and LH. Oestrogen secreted by the maturing follicles feeds back onto the hypothalamic pituitary axis to regulate FSH/LH secretion by the pituitary gland and ultimately triggers the ovulatory surge of LH which causes ovulation. A corpus luteum is formed, which secretes progesterone, which in turn suppresses stimulation of the hypothalamus pituitary axis by oestradiol. The corpus luteum in the rat, only becomes fully functional under the influence of prolactin in pregnancy or pseudo-pregnancy.

to grow and the time when it ovulates there is synchronized development of the oocyte and the follicle. Once a follicle enters the proliferating pool of follicles, it is committed to continuous growth which eventually terminates when that follicle ovulates or becomes atretic, (Peters, 1976). In a single oestrous cycle, the majority of developing antral follicles undergo atresia. A follicle which is destined to ovulate must be protected from the normal degenerative process of atresia (Condon, Ganjam, Kenny and Channing, 1979). The growth of preantral and antral follicles to the point of ovulation and the regulation of atresia will be discussed later.

1.2.1 Follicular development from the stage when oocytes can be identified to the stage when follicles enter the pool of non-growing follicles

Our understanding of the development of follicular organization has come largely from studies in the mouse. In this species, no follicles are formed before birth, (Dawson and McCabe, 1951). "At birth the oocytes are situated within the intra-ovarian cords of the rete ovarii." During the first two postnatal weeks, cells from the rete "become attached to the surface of the oocyte" and establish the first granulosa cell layer (Byskov and Lintern-Moore, 1973). As the follicles are forming, "there are open (cytoplasmic) connections between the follicles and the rete tubules". But at a later stage the basement membrane which surrounds the oocyte and the cells which have become attached to it, becomes a continuous entity and the follicles become independent units. (Byskov and Lintern-Moore, 1973). These units, which consist of the

oocyte, the granulosa cells and the basement membrane surrounding them, constitute the pool of non-proliferating follicles from which the developing follicles emerge when growth is resumed (Peters, 1976).

Very little is known about the non-proliferating small follicles and their precise influence on ovarian physiology. However, we do know that the size of this pool influences the number of follicles which begin to grow during infancy: a reduction in the size of this pool is followed by a reduction in the number of follicles which start to grow (Krarup, Pedersen and Faber, 1969). The reduced size of the pool can be the result of ageing or it can be reduced at an early age by exposing the animal to a single dose (20 rads) of radiation, to a single oral dose of 9:10 dimethyl 1:2 benzanthracene (DMBA), or by injecting testosterone propionate. All these manipulations of the size of the pool of non-proliferating follicles are followed by a subsequent reduction in the number of developing follicles (Peters, 1969; Krarup, Pedersen and Faber, 1969; Peters, Sorensen, Byskov, Pedersen and Krarup, 1970). How this control is mediated is at present unknown. The small follicles could act via a feedback mechanism operating through the hypothalamic - pituitary system and FSH secretion. The small follicles may secrete an 'inhibin'-like substance, influencing FSH secretion, which in turn may influence the developing follicles. Alternatively the control of the size of the pool of non-proliferating follicles could operate through local influences within the ovary. It has been demonstrated that there is follicular hyperplasia in mice following hemi-ovariectomy, suggesting that a negative

feedback system may operate (via the hypothalamic pituitary axis) at a very early age (Peters and Braathen, 1973). However, the small follicular pool size is not the only factor which regulates the number of developing follicles. For example, the number of follicles which start to grow per 24 h differs during maturation in the mouse (Pedersen, 1972). The very high rate of growth - initiation during the first postnatal week is approximately halved three weeks later and remains more or less constant in the mature cycling animals (Table 1). The fact that only half as many follicles start to grow in three week old animals as in one week old animals suggests that in the intervening time a factor might act which impedes growth initiation (Pedersen, 1969). It was postulated that the number of follicles which are undergoing atresia might influence the number which are leaving the pool of non-growing follicles. It was suggested that a factor might be present in the follicular fluid of follicles which could influence the rate at which follicles start to grow (Peters, Byskov and Faber, 1973). A factor with this property was identified when Peters et. al., (1973) demonstrated that injections of follicular fluid from large bovine follicles subcutaneously into neonatal mice resulted in a reduced rate of initiation of the growth of follicles. The factor(s) involved in slowing down growth initiation has not yet been identified.

A tentative simplified model of follicular growth is shown in figure 1.2.

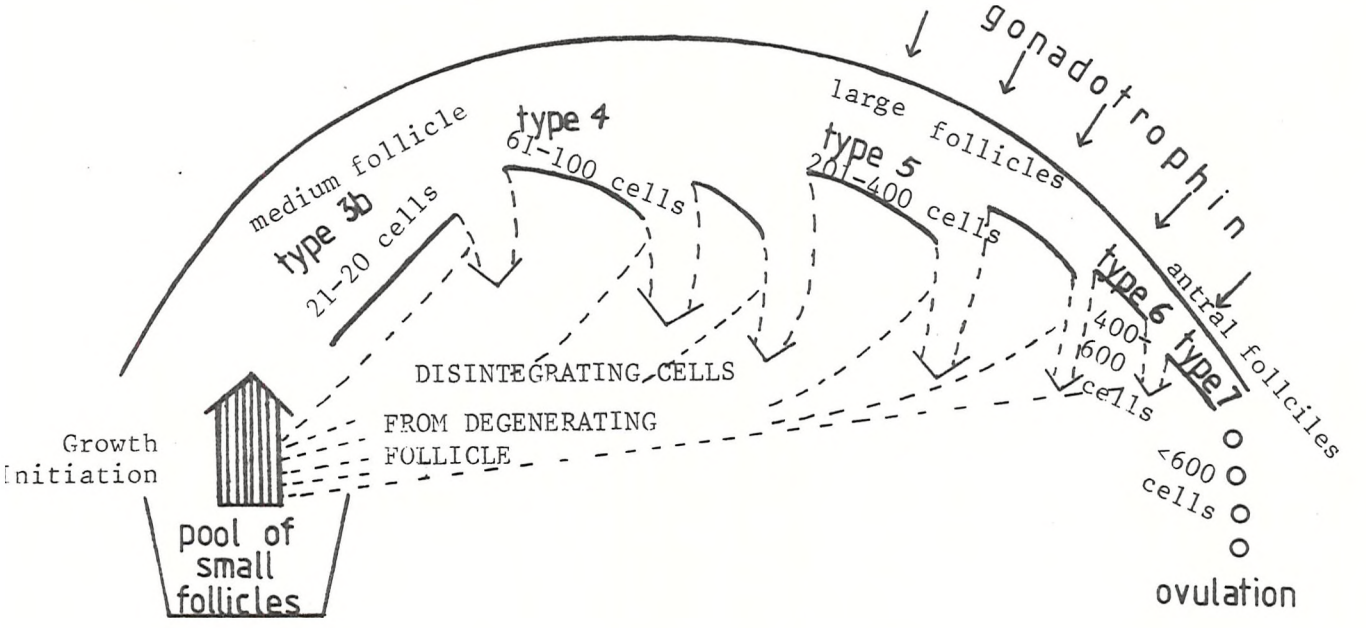
Table 1. Number of follicles starting to grow in the mouse ovary per 24 h period at different ages (Pedersen, 1972).

Weeks					Months				
1	2	3	4	5	3	3*	9	12	16
41	31	21	20	17	19	14	10	10	3

*Pregnant

The very high rate at which follicles start to grow during the first week of life falls to half this level by 3 weeks of age and remains approximately constant in the mature cycling animals.

FIGURE 1.2 SIMPLIFIED MODEL OF FOLLICLE GROWTH.



During the first week of life very few follicles with degenerating cells and none with antral fluid are present in the ovary, while the rate of growth initiation is high. As the number of growing follicles and large follicles increases, the number of degenerating follicles also increases. Subsequently, the rate of growth initiation is reduced. (Adapted from Peters et al., 1973).

1.2.2 The growth of follicles from the pool of non-proliferating follicles to the stage of antrum formation: their dependence upon gonadotrophins

The growth of a follicle involves four integrated events: the oocyte (previously in the dictyate stage) starts to enlarge; the zona pellucida is formed around the oocyte; the granulosa cells increase in number by mitosis, forming several layers; and thecal layers differentiate. A single hypothesis which integrates the hormonal and/or local factors which regulate these early events has yet to be formulated. However, it has been suggested by Richards and Midgley (1976) that thecal cells might differentiate in response to a theca cell 'organizer' produced by the granulosa cells. Cells that can be identified as thecal cells appear early during follicular growth. Follicles with a growing oocyte and one layer of granulosa cells (Stages 3a to 4 as defined by Peters, (1969)), often lack a complete thecal envelope but show cells which lie in close proximity to the basement membrane, which delineates the granulosa cells. These are the earliest stages of cellular differentiation for cells which eventually form the thecal layer. In the early preantral stages of follicular development, the thecal cells become well defined. At later stages (Stages 7 and 8 as defined by Peters, 1969), as the antrum forms, thecal cells increase in size and in number. In some species, like the hamster, the thecal layer develops early and is almost as wide as the granulosa layer. It has been suggested that one of the reasons why an understanding of the control of differentiation of the thecal layer has been slow to materialize, is that this layer is potentially sensitive to and dependent upon the interplay of hormones for its successful development

(Peters, 1979).

The fundamental role played by the pituitary gonadotrophins - Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and perhaps also by prolactin (PRL) in stimulating follicular development in prepubertal and adult animals has been extensively investigated. The reported effects of FSH and of LH on follicular maturation during the first 18 days of life in rodents are controversial (Schwartz, Andersen, Negiun and Ely, 1974). In these animals, ovarian development was formerly thought to be independent of pituitary hormones during the period between birth and a few days before weaning (Hertz, 1963). Hertz reached this conclusion following experiments in which an ovary from new born rats was grafted under the kidney capsule of hypophysectomized adult males. The grafted ovary underwent a complete morphological differentiation in the ensuing 21 days (weaning occurring in about 21 days). From this observation one might deduce that the steroids secreted at this stage were not controlled by the secretion of pituitary gonadotrophins. However, studies in which antisera against rat gonadotrophins were injected into mice suggest that early stages of follicular growth depend on gonadotrophins (especially FSH), since following the injection of FSH - antisera the ordered growth of follicle is disturbed. The results of these experiments, however, are controversial. Thus mice which had been injected with an antiserum against rat pituitary gonadotrophins during the first 14 days of life, showed an abnormal development of the granulosa layer and a lack of thecal development, which was corrected by injecting exogenous FSH or human menopausal gonadotrophin (HMG), (Eskhol, Lunenfeld and Peters, 1970). Similar experiments in rats

failed to alter granulosa and thecal cell development but led to a deficient interstitial cell development in the antiserum treated rats (Schwartz, Andersen, Nequin and Ely, 1974).

In a comparable study, Uilenbroek, Wolff-Exalto and Welschen (1976) investigated whether the high FSH levels present before day 20 in female rats (Ojeda and Ramirez, 1972; Meijs-Roelofs, et al. 1973) are of physiological importance for normal follicular development. The effect of suppressing FSH levels on the numbers of large follicles was studied. It was observed that injecting an antiserum, which had been raised against FSH, from day 7 till day 11 resulted in a decreased number of large follicles on day 12.

The foregoing observations tend to support the view that the early stages of follicular development are dependent upon gonadotrophins (especially FSH). Similar results have been obtained from studies in vitro. It has been shown, for example, that the addition of pregnant mares' serum gonadotrophin (PMSG), or FSH to organ cultures of ovaries of prepubertal rats and mice, increases the number of granulosa cells undergoing mitosis, and also the number of medium sized follicles (Fainstat, 1968; and Ryle, 1969). While antral formation was stimulated in ovaries exposed to both FSH and LH (Ryle, 1970). Thus dependence upon gonadotrophins already exists in the preantral stages of follicular development, and persists through to the preovulatory stage.

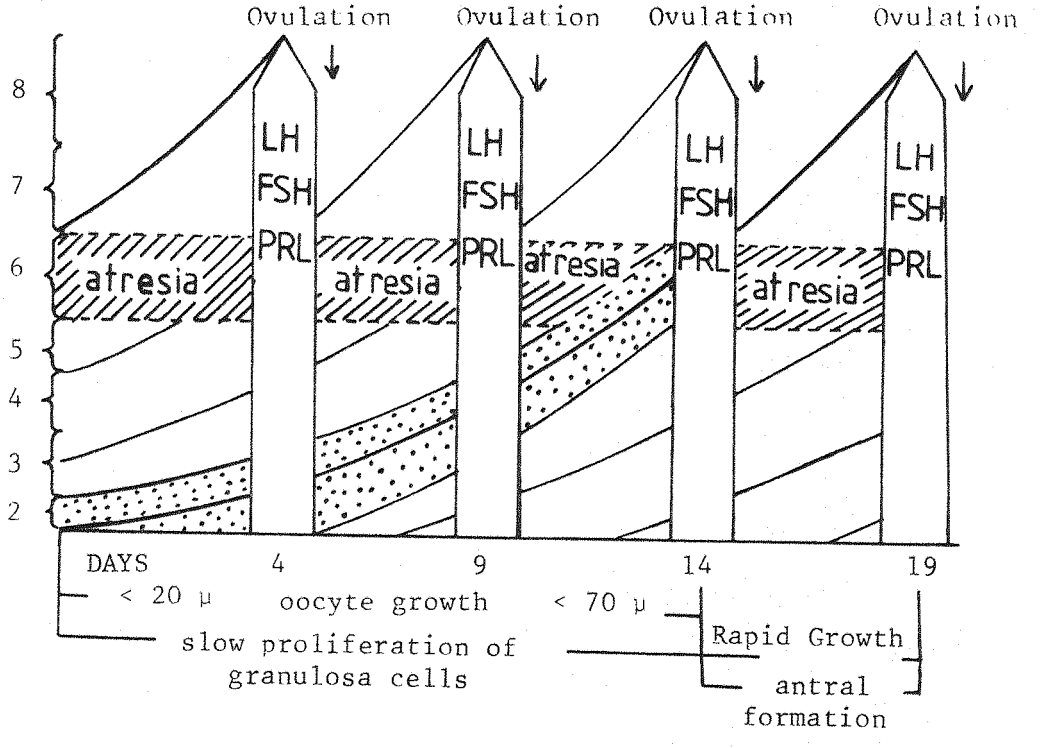
1.2.3 Continued antral follicular development: dependence on gonadotrophins

The requirement for gonadotrophins by antral follicles is absolute. This was clearly demonstrated in experiments in

which immature rats were hypophysectomized. Hypophysectomy resulted in atresia (death) of antral follicles (Mauleon 1969; and Schwartz and McCormack, 1972). The role of atresia in follicular development will be fully discussed in a later section.

Follicular development in prepubertal animals has been extensively studied because there is a continuum of follicular development which is not influenced by the presence of large preovulatory follicles or by the endogenous LH surge. A model of the dynamic changes in the population of growing follicles during the oestrous cycle of the rat is illustrated in figure 1.3. The data has been derived primarily from studies in the mouse (Pedersen, 1970; Pedersen and Peters, 1970) and rat (Hirsfield and Midgley, 1978). The different stages of growth (represented on the vertical axis) are based on the classification of Pedersen and Peters, (1968). The follicles are subdivided into groups: small follicles (Type 2), first entering the pool of committed follicles. These follicles progress through to the stage of large preantral follicles (Type 5 and 6); and thence to large antral, preovulatory follicles (Type 7 and 8). It can be seen from the diagram (Fig. 1.3) that some follicles (represented by solid lines) start to grow asynchronously each day and continue to grow until atresia or ovulation occurs. "Thus, these follicles which ovulate in a given cycle, such as those represented by the solid line (-), actually began to grow 19 days earlier as a part of a large pool of growing follicles, represented by the stippled area. Most of these follicles continue to grow until, as large preantral follicles, they undergo atresia, as

FIGURE 1.3 PRESUMPTIVE PATTERN OF FOLLICULAR GROWTH DURING FOUR OESTROUS CYCLES IN THE RAT.



The diagram presupposes that a given number of follicles (stippled area) begins to grow each day and continues to grow until either atresia (hatched areas) or ovulation (solid line) occurs. On any given day, such as oestrus, many stages of development are present. Note, however that no large antral follicles are observed at oestrus. In contrast, on pro-oestrus one would predict from the diagram that large antral follicles but no small antral follicles will be present. Recent observations in cycling rats by Hirshfield (1976) support this pattern of the final growth of follicles and have indicated further that the FSH of one cycle stimulates the growth of those follicles destined to ovulate at next cycle. (From: Pedersen and Peters, 1968; Richards and Midgley, 1976; Richards, 1979).

indicated by the hatched area, only a few follicles enter the final stages of follicular growth (Type 7 and 8)" (Richards, 1978). The selection of the follicles from the pool of large preantral follicles has been associated with the FSH surge (Schwartz, 1974).

1.2.4 Continued follicular development to the stage of ovulation: dependence upon gonadotrophins

The regulation of the development of preovulatory follicles in the rat is absolutely dependent upon changes in gonadotrophin secretion which occur during the oestrous cycle. A 'surge' of both FSH and LH occurs late in the afternoon of pro-oestrus, the FSH surge extending into the morning of oestrus (Smith, Freeman and Neill, 1975). Follicles which ovulate in response to the gonadotrophin surge of one cycle, as has been said, began to grow 19 days earlier as a part of a larger pool of growing follicles. Thus, once committed to grow a follicle is exposed to at least three consecutive surges of gonadotrophins (Richards, 1978). It has been proposed that the surge which preceeds ovulation selects from a growing pool those follicles which will ovulate and luteinize at the subsequent pro-oestrus (Welschen, 1973; Schwartz, 1974; Richards and Midgley, 1976). Those follicles which are not selected or are not capable of responding to the gonadotrophin signal become atretic and fail to mature. Thus the surge of LH and FSH at pro-oestrus appears to have two primary functions: FSH dictates that a selected number of follicles enter into the final stages of growth, while LH terminates follicular growth and initiates ovulation (Welschen, 1973). LH may also cause atresia around the time of ovulation.

On the basis of this discussion, it would appear that follicles are unequally responsive to gonadotrophins and that the state of differentiation of the follicular cells (both granulosa and theca) determine which follicles will respond to the surges of gonadotrophins and enter the final phase of follicular growth. The granulosa cells of most follicles, even those with one or two layers of granulosa cells, appear to possess the postulated receptor sites for FSH which should be responsible for mediating the FSH responses (Eskhol and Lunenfeld, 1972; Richards et al., 1976). However, only the granulosa cells of large preovulatory follicles, also possess postulated receptor sites for LH which should be responsible for mediating the LH responses (Channing and Kammerman, 1974; Zeleznik, Midgley and Reichert, 1974). It seems reasonable in the light of these observations, as has been proposed, that changes in the ability of follicles to take up the gonadotrophins might determine the response of these follicles to these trophic hormones.

Furthermore, interactions between the gonadotrophins and changes in the sensitivity of the follicles have been demonstrated. Serum levels of gonadotrophin during the oestrous cycle of the rat are low from met-oestrus to early pro-oestrus (Richards, 1978). However, if exogenous gonadotrophins (PMSG/HCG) are administered during this period, ovulation can be stimulated on di-oestrus but not during oestrus (Welschen, 1973). From this, one can conclude that follicular growth appears to be associated with an increased responsiveness by the follicles to the gonadotrophins.

1.3 THE ACTION OF GONADOTROPHIN ON OVARIAN FOLLICLES: A KEY TO UNDERSTANDING OVARIAN DEVELOPMENT AND FUNCTION

Before one can comprehensively discuss the regulation by gonadotrophins of the growth of follicles, and of steroid secretion, one has to understand current concepts about the mechanism of action of gonadotrophins.

Many of our present ideas about LH/HCG action have been derived from studies on Leydig cells (Catt and Dufau, 1973; Means, 1973) and corpora lutea (Marsh, Butcher, Savard and Sutherland, 1966; Marsh, 1970). A scheme for the mechanism of action of LH on ovarian cells is shown in figure 1.4. The first step requires that the target cell, in this case thecal cells, specifically interact with the trophic hormone. Thus LH binds to a specific receptor site on the surface of the target cell (Lee and Ryan, 1972; Gospadarowicz, 1973). This results in the activation of the membrane bound enzyme adenylate cyclase and thence the production of adenosine 3':5' - cyclic monophosphate (c'AMP). This leads to a rise in the intracellular concentration of c'AMP (Mason, Schaffer and Toomey, 1973; Lindner, Tsafriri, Lieberman, Zor, Koch, Bauminger and Barnea, 1974). It is postulated that the c'AMP interacts with the regulatory subunit of a protein kinase. This results in a dissociation of the subunits and an 'un-masking' of the activity of the catalytic subunit (Figure 1.5), (Vaitukaitis and Albertson, 1979).

In a series of subsequent events, which have yet to be defined, steroidogenesis is stimulated (Marsh, 1976; Channing and Tsafriri, 1977). Some of the steps which may be involved in this sequence are shown in figure 1.4. They are:

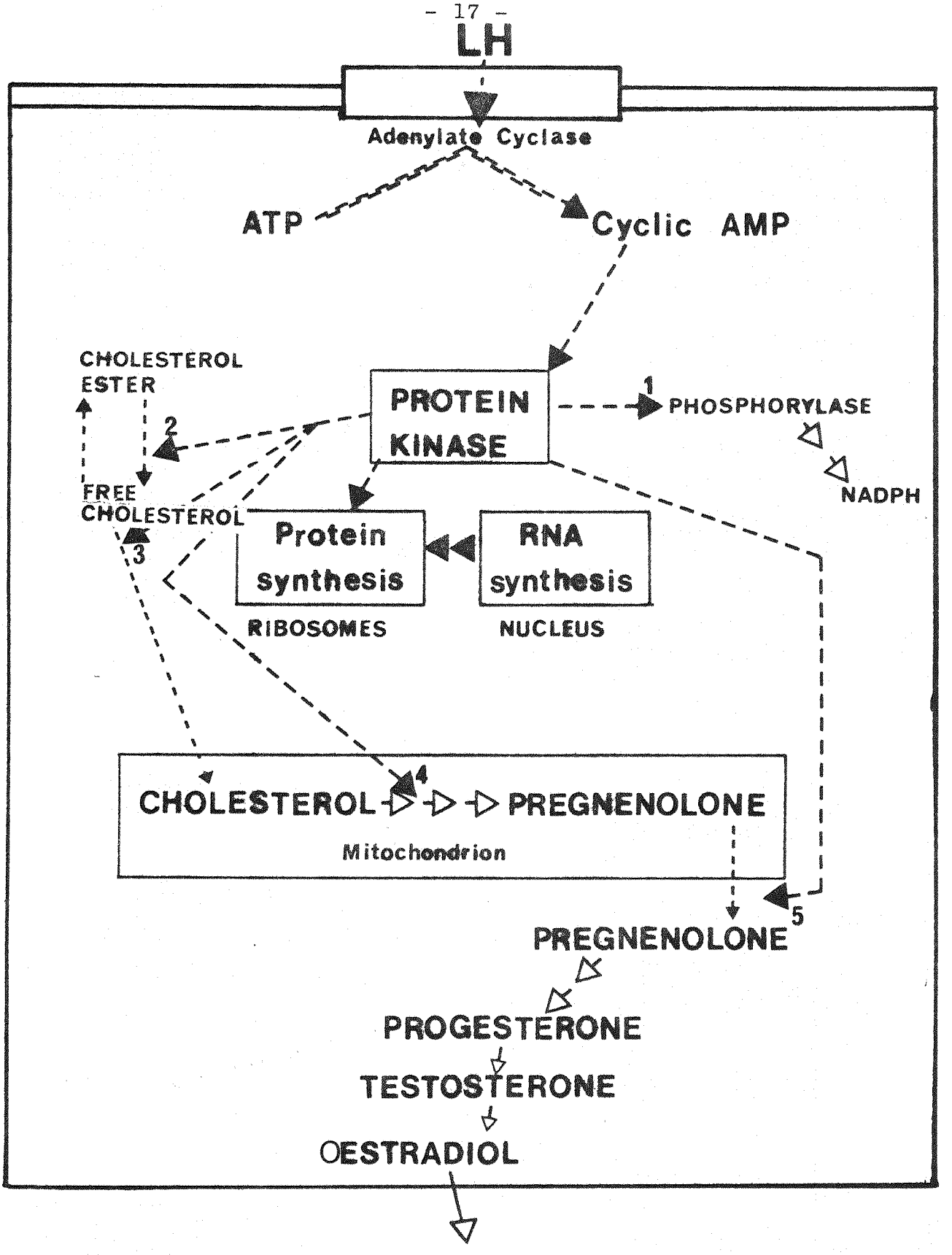
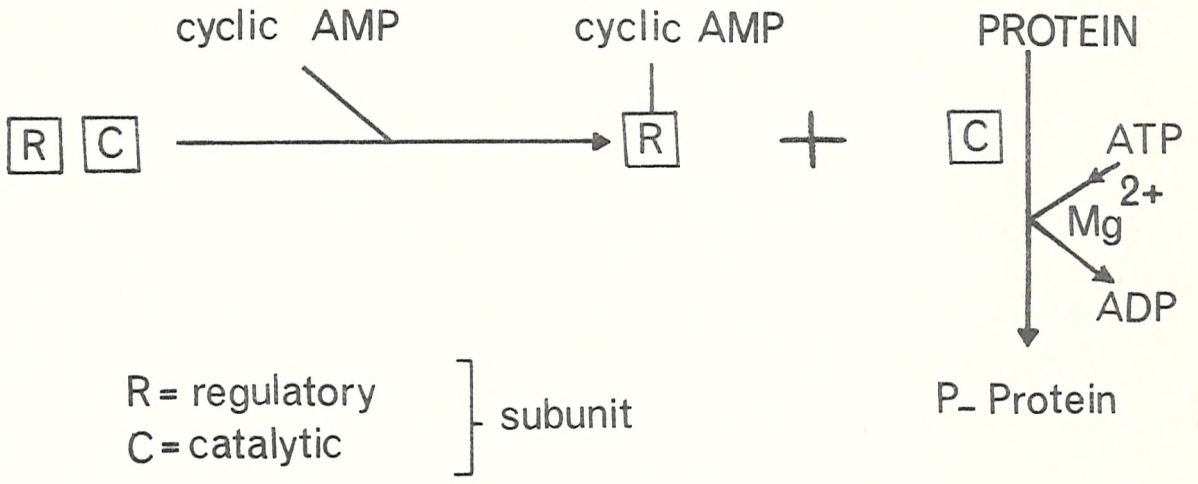


Figure 1.4 Possible sites of action of cyclic AMP on steroid secretion in a hypothetical gonadal cell. ⇝ , established biochemical reactions; - - → , transport of substances through membranes of the cell. Five possible protein kinase-mediated effects of c'AMP on steroid biosynthesis are shown. The numbers (1-5) refer to the postulated sites of the effects of increased protein kinase activation enunciated in the text. (adapted from Marsh, 1976).

FIGURE 1.5 THE MECHANISM BY WHICH c'AMP BRINGS ABOUT ACTIVATION OF PROTEIN KINASE. (ADAPTED FROM RASMUSSEN, 1974; VAITUKAITAS AND ALBERTSON, 1979).



c'AMP activates protein kinase by binding to the regulatory subunit (R) of the inactive enzyme (RC), thereby releasing the active (c'AMP-insensitive) catalytic subunit (C) which catalyzes the transfer of phosphate from ATP to cellular proteins.

1) Increase in co-factor availability,

2) Increase in substrate availability,

3) Facilitation of cholesterol transport,

4) Increase in side-chain cleavage activity, or

5) Improved efflux of pregnenolone from the mitochondria.

The mechanism of action of FSH on the ovary of the rat has not been as extensively studied as the action of LH and will only be discussed briefly here. In the rat ovary, the FSH receptor is located exclusively on the granulosa cells (Zeleznik et al., 1974). As in the case of LH already discussed, FSH stimulates steroidogenesis by activating a specific FSH-sensitive adenylate cyclase, which results in an increase in the intracellular c'AMP concentration (Lindner et al., 1974). The c'AMP produced is thought to initiate a complex sequence of events which results in the induction or activation of one or more of the rate-limiting enzymic steps in steroidogenesis (Kolena and Channing, 1972; Marsh, 1976). However, many of the effects of FSH are observed only after several hours or even days of exposing the cells to FSH. Therefore the effects of LH and FSH may ultimately be found to operate at different stages of steroidogenesis. The steps which have been suggested as the mechanism of action of gonadotrophins have not included the role of protein synthesis, although it is known that protein synthesis is an integral component of the response system. For example, some of the unknown stages are: which proteins are formed; are the enzymes activated; or does <u>de novo</u> synthesis of whole enzyme complexes occur?.

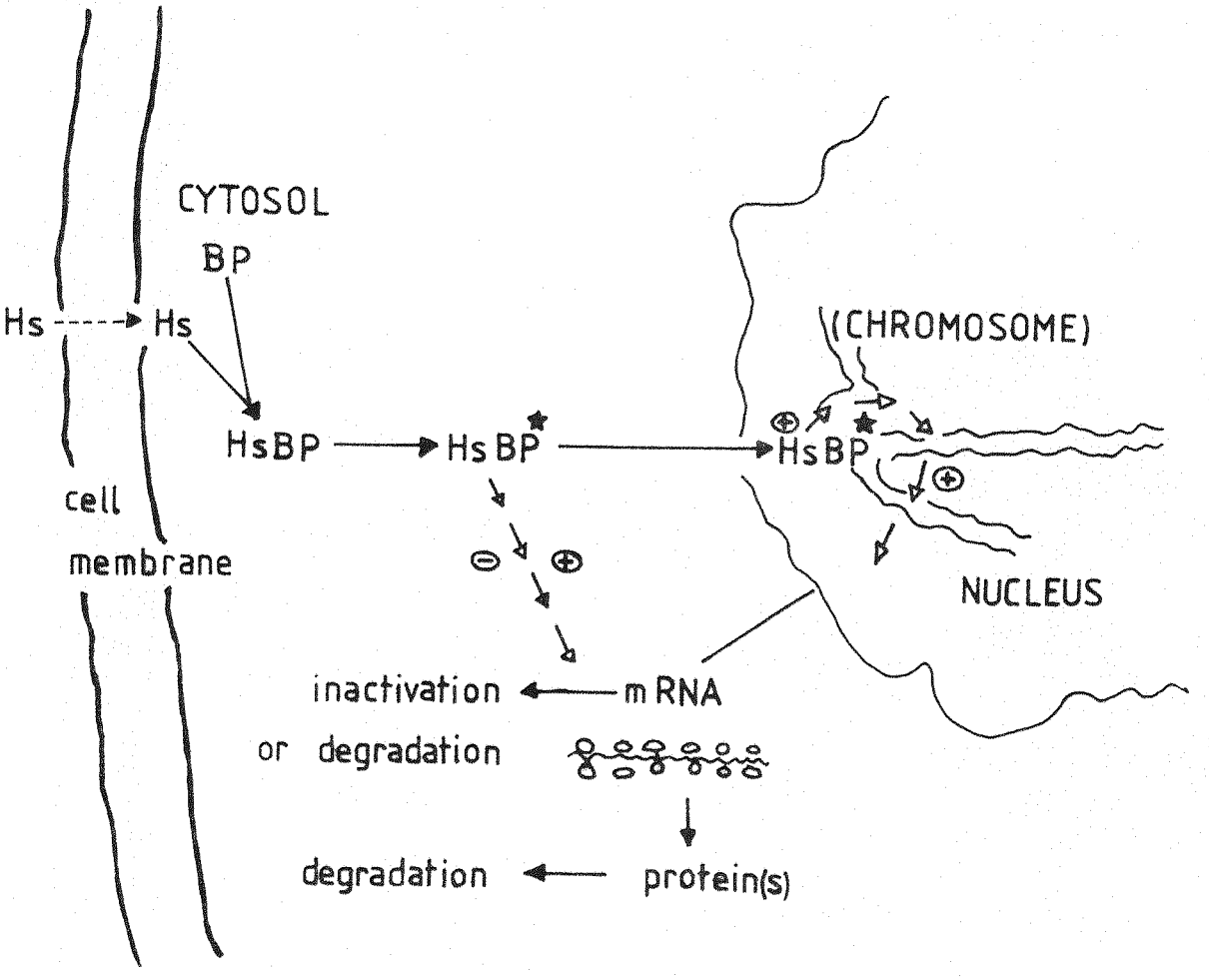
The above discussion centres on the postulated models for the mechanism of action of gonadotrophins (LH and FSH) in the rat's ovary. Since the regulation of the growth of follicles also involves steroid hormones, as will be shown, it seems reasonable to discuss the postulated models for the mechanism of action of steroid hormones.

1.3.1 Mode of action of steroid hormones - oestrogen as a prototype

The prototype for the general model of steroid hormone action which has been selected is that of oestrogen and its effect on the growth and development of the mammalian uterus or avian oviduct (Rasmussen, 1974; Muller and Cowan, 1974; King and Mainwaring, 1974). The combined data from these two model systems is the basis for the model of oestrogen action described in Fig. 1.6.

Firstly, the oestradiol-17β (E_2-17β) enters the cell cytosol. In the cytosol, the oestradiol binds to a specific receptor protein (BP). Following this hormone-receptor interaction, the receptor-protein complex undergoes a structural modification and is then transported, as a steroid-protein complex, into the nucleus. Within the nucleus, the oestradiol-receptor complex interacts with one or more specific parts of the genome. This initiates the activation of DNA polymerase and thus the transcription of specific genes, e.g. the gene for ovalabumin synthesis in the avian oviduct. More than one type of nuclear receptor has been identified, which may have different physiological roles. The control of many of these events which regulate the rate of oestrogen stimulated RNA/protein systhesis have yet to be investigated. They are however

FIGURE 1.6 POSTULATED MODEL FOR THE MECHANISM OF ACTION OF OESTRADIOL IN OESTROGEN-DEPENDENT TISSUES (ADAPTED FROM RASMUSSEN, 1974)



The first step in the activation of the cell by oestradiol-17β is the entry of the steroid (H_S) into the cell cytosol. There it binds to a specific receptor protein (BP). Following this hormone-receptor (H_SBP) interaction, the receptor-protein undergoes a structural modification and is transported, as a steroid protein complex (H_SBP), into the nucleus where it is thought to bind, with non-histone protein, to the genome at specific locations. The resulting activation of RNA-polymerase results in transcription of the genome into newly formed RNA. Subsequently, by mechanisms unknown, the oestradiol is lost from the nucleus. (mRNA = messenger RNA).

fundamental to a complete appreciation of these important events.

1.3.2 The role of oestradiol and follicle stimulating hormone in regulating follicular cell functions

The observation that exogenous oestrogen increased the responsiveness of rat ovarian follicles to gonadotrophins (Pencharz, 1940; Williams, 1940; Smith and Bradbury, 1963) led to the suggestion that the follicular production of oestrogen and/or the response of follicular cells to oestrogen may affect the rate of follicular growth and the differentiation of follicular cells (Richards and Midgley, 1976). Studies with hypophysectomized immature female rats given graduating doses of diethylstilboestrol (DES) have shown that oestrogen stimulates ovarian weight and that this response is due to a reduction in atresia of the preantral follicles and a concomitant stimulation of preantral follicular growth (Payne and Hellbaum, 1955). Goldenberg, Vaitukaitis and Ross (1972), demonstrated a synergistic effect between FSH and DES on ovarian weight gain when graduated doses of FSH were administered following 2 - 4 days of graduated doses of DES. A predominance of large preantral follicles was demonstrated in ovaries of animals pretreated with DES alone whereas ovaries from animals treated with both hormones (DES and FSH) contained numerous antral follicles. The mechanism(s) through which oestrogen exerted this effect included an increased ovarian uptake of labelled FSH (Goldenberg et al., 1972).

The interactions between oestradiol and FSH which regulate granulosa cell functions have been re-examined by Richards

(1978), using the hypophysectomized female immature rat as an experimental animal. This model has no endogenous gonadotrophins so that responses to exogenous trophic hormones can be recognised more readily. (But it has no growth hormone or ACTH/corticosterone secretion, so that in some respects results are open to circumspection). Rats were hypophysectomized at 24 days of age and treated with oestradiol (1.5 mg/day for 4 days) followed by highly purified preparation of hFSH (2 µg/day for 2 days). This treatment stimulated the development of large preantral and antral follicles (Richards, 1978).

The effect of these hormones on granulosa cell proliferation was examined. It was found that oestradiol increased the proliferation of granulosa cells as indicated by calculating the DNA content of isolated granulosa cells or by estimating the percentage of 'labelled' cells observed following in vivo administration of 3H-thymidine and subsequent analysis by autoradiography (Rao, Midgley and Richards, 1978). However, despite the continued treatment with oestradiol, granulosa cell proliferation was not sustained longer than 24 hours as indicated by the decreasing labelling index of these cells, and by the plateau in the granulosa cell DNA content. If, however, FSH was given to oestradiol treated rats, a new wave of proliferative activity followed within 24 h, which again levelled off after 48 h.

The effects of oestradiol and FSH on receptors for FSH and LH show that oestradiol alone caused only a slight increase in the content of FSH receptor per granulosa cell in this preparation. Richards argues that such a modest increase in the

number of FSH receptors would not be sufficient to account for the marked increase in the responsiveness of oestradiol-primed granulosa cells to FSH (Richards, 1979). This, she says, shows that the hypothesis that oestrogen might act to increase the number of FSH receptors per granulosa cells is invalid. In contrast, hFSH alone caused a progressive and a substantial increase in the number of FSH receptors per granulosa cell (Richards, 1979). Taken together, these results appear to indicate that FSH, more than oestradiol, acted to increase the number of receptors for FSH. However, FSH alone appeared to have little effect on the number of LH receptors per granulosa cell, but it required both oestradiol and FSH to induce LH receptors (Richards, 1978). These results with hypophysectomized immature female rats differ from observations in intact immature female rats, in which rat FSH alone greatly increased the LH receptors localized in the granulosa cells of developing follicles (Zeleznik et al., 1974). It must be noted, however, that in intact immature rats, oestradiol would have been present in the ovaries. However, exactly how oestradiol and FSH interact to stimulate granulosa cell differentiation is still unanswered. To examine this question, many investigators have examined components of the FSH response system; namely the stimulation of c'AMP production and the regulation of the intracellular concentrations of c'AMP regulatory subunits (Hunzicker, Dunn, Jungmann and Birnbaumer, 1979).

There is considerable experimental evidence indicating that many of the effects of FSH and LH on ovarian follicles are mediated by c'AMP, as reviewed by Marsh (1975). These

gonadotrophin-induced elevations in c'AMP content seem to be due to the activation of the membrane bound adenylate cyclase (Kolena and Channing, 1972) rather than to changes in the rate of catabolism of c'AMP by phosphodiesterase (Hunzicker-Dunn et al., 1979). FSH-stimulated adenylate cyclase activity in small antral follicles i.e. follicles obtained from cycling rats on the morning of oestrus and in follicles obtained from PMSG-primed rats, 24 h after the injection of this hormone, is considerably higher than the LH-stimulated adenylate cyclase activity (Hunzicker-Dunn et al., 1979). Similarly Koch, Zor, Pomerantz, Chobsieng and Lindner (1973) reported that highly purified FSH (50 µg/ml), but not LH (5 µg/ml) promotes an 11-fold increase in the concentration of c'AMP of ovaries obtained from 27- to 29-day old rats (these ovaries presumably contain only small antral follicles). Thus, there is substantial evidence indicating that the FSH-responsive adenylate cyclase system is in fact mature in small follicles (at least in the rat), and that it is coupled functionally to FSH receptors (Hunzicker-Dunn et al., 1979).

In addition to the roles of FSH already mentioned, FSH is capable of activating or producing granulosa cell aromatase activity in vivo and in vitro (Dorrington, Moon and Armstrong, 1975; Erickson and Hseuh, 1978; Hillier, Zeleznik, Knazek and Ross, 1980; Hillier et al., 1980a). Histochemical and microscopic studies of steroidogenic enzymes showed that FSH stimulated 3β-hydroxy-steroid dehydrogenase activity in granulosa cells of the immature rat, while in the adult cycling rat the activity of this enzyme reached a maximum in preovulatory follicles obtained at pro-oestrus (Pupkin, Bratt, Weisz, Lloyd and Balogh, 1966). Taken together, these ob-

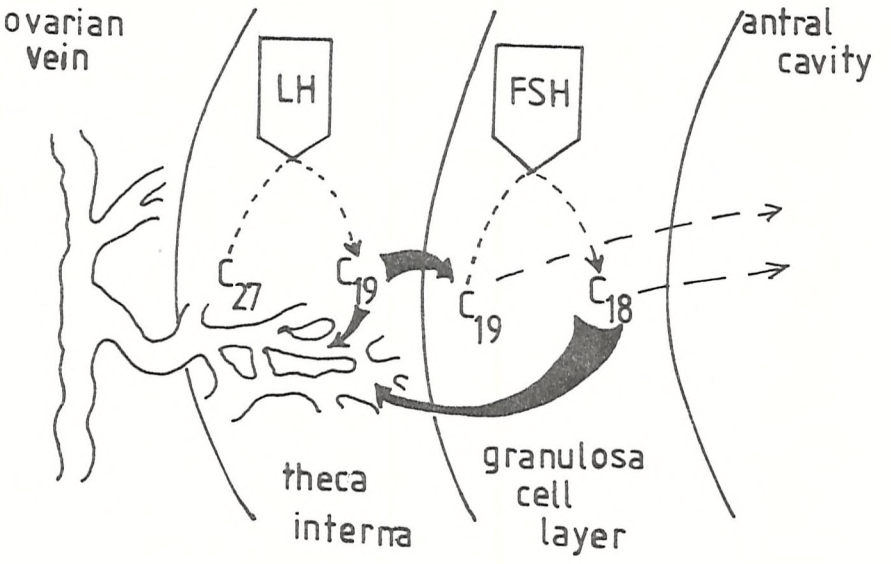
servations seem to imply that FSH may be responsible for the induction or activation of at least one of the enzymes present in the granulosa cells of preovulatory follicles (Hillier, et al., 1980b).

The preceding discussion also indicates the importance of oestrogen in the early stages of normal follicular development and the effect which oestradiol has in facilitating follicular responses to FSH. Although the principal cellular source of oestrogen in the ovarian follicle has yet to be resolved, preovulatory Graafian follicles are thought to be responsible for the large amounts of oestrogens secreted by the ovary into the "peripheral circulation during oestrus" (Makris and Ryan, 1975; Hillier 1981). The experiments of Falck (1959) and more recent investigations by Makris and Ryan (1975) indicated that both granulosa cells and thecal tissue were needed for ovarian oestrogen synthesis. Attempts have been made to determine the contribution of each cell type (theca and granulosa cells), but the investigations have been hampered by the difficulty in obtaining a pure thecal preparation free from granulosa cells (Hansel and Fortune, 1978). Various models for follicular steroidogenesis have been proposed. One hypothesis holds that the theca is responsible for androgen and oestrogen synthesis and is the principal source of these steroids in preovulatory follicles, while granulosa cells are responsible for the high progesterone production occurring just before ovulation. This observation was made in the mare (Short, 1964). However, subsequent investigations in the rat have produced an alternative scheme for the site(s) of oestrogen biosynthesis. Armstrong and Dorrington (1977); Hiller, (1981), produced

convincing evidence which supports a "two cell, two gonadotrophin hypothesis'. They proposed that the theca, under the influence of LH, secretes androgen which is aromatized to oestrogen by granulosa cells which have been stimulated previously by FSH (Fig. 1.7). These two models thus disagree in the cellular site of aromatization. However, these differences may be due to species variation. In both monkey and humans, for example, it has been shown that the theca makes a substantial contribution to oestrogen secretion (Ryan, Petro and Kaiser, 1968; Channing and Coudert, 1976; McNatty, Makris, De Grazia, Osathanondh and Ryan, 1979). Much of the evidence relating to the site(s) of oestrogen synthesis has been reviewed recently (Ryan, 1979, and Hillier 1981).

Experiments in which cells were isolated from follicles of hamsters and rats at pro-oestrus have shown that thecal but not granulosa cells secrete aromatizable androgen (testosterone and androstenedione) and that the secretion of androgens is stimulated by LH in vitro (Makris and Ryan, 1975; Fortune and Armstrong, 1977). In addition, Dorrington et al., (1975) showed that FSH but not LH, stimulated the secretion of oestradiol from granulosa cells isolated from preantral follicles when the cells were incubated with aromatizable substrate, testosterone. This effect of FSH on the secretion of oestradiol by granulosa cells was only observed after the cells had been exposed to FSH for 24 h, while there was no immediate response (within 6 h) to this gonodatrophin. More recently, theca and granulosa cells isolated from pro-oestrus rats' follicles were cultured with highly purified LH and/or FSH in the presence or absence of testosterone. In the absence of

FIGURE 1.7 CURRENT CONCEPTS OF THE CONTROL OF OESTROGEN BIOSYNTHESIS IN THE PRE-OVULATORY FOLLICLE BY LH AND FSH.



Androgens (C_{19}) are synthesized from cholesterol (C_{27}) in the vascularized theca interna under the influence of LH. The avascular granulosa cell layer, under the influence of FSH, acquires an active aromatase enzyme system. The androgen diffuses across the lamina basalis to serve as the substrate for aromatization. Cells in the parietal granulosa cells are thought to be the most active sites of aromatization. The cells are adjacent to the rich network of blood vessels which 'encapsulates' the lamina basilis. The oestrogen (C_{18}) they produce diffuses into the blood stream. Some steroid, also passes into the antral cavity (adapted from Hillier, 1981).

testosterone, both theca and granulosa cells synthesized low levels of oestradiol. The addition of testosterone to the culture medium resulted in an enhanced secretion of oestradiol by the granulosa cells and only a slight increase in the oestradiol secreted by thecal preparations (Fortune and Armstrong, 1978; Armstrong, Goff and Dorrington, 1979). However, histological examination revealed that the thecal preparations contained some contaminating granulosa cells. When great care was taken to remove the granulosa cells, the amount of oestradiol secreted by the presumptive thecal cells was very small indeed (Fortune and Armstrong, 1978). They therefore suggested that under normal circumstances thecal cells do not contribute to the net secretion of oestradiol by rat follicles.

The mechanisms which control the conversion of aromatizable androgens to oestrogens have been examined. The observation of Erickson and Ryan (1975) that dibutyrl c'AMP stimulated oestradiol secretion by cultured rabbit granulosa cells raised the possibility that c'AMP may be an intracellular mediator of the action of FSH. This observation led Armstrong et al. (1979) to investigate the ability of FSH to stimulate c'AMP production by isolated granulosa cells. Their results demonstrated the effectiveness of purified FSH in stimulating c'AMP production by granulosa cells from intact, oestrogen-primed immature rat. Although they observed a slight elevation in intra-cellular levels of c'AMP when the cells were exposed to LH, this occurred only at the maximum concentration of the hormone (500 ng/ml). At this high level of exogenous LH, the effects of contaminating FSH could not be excluded. These results are consistent with the hypothesis that FSH stimulates granulosa cell aromatase

activity through activation of a hormone-sensitive adenylate cyclase enzyme (Armstrong et al., 1979). Evidence obtained with isolated testicular Sertoli cells has led to a similar conclusion concerning the mechanism of action of FSH (Dorrington, Fritz and Armstrong, 1978).

These observations suggest that LH and FSH regulate ovarian oestrogen secretion by actions at biochemically distinct sites: LH stimulates the synthesis of androgens in the theca interna cells which are then converted to oestrogens in the granulosa cells under specific stimulation by FSH.

1.3.3 The role of luteinizing hormone and androgens in follicular cell functions

Some effects of LH on the early stages on follicular development have been described already. The histological studies of Lostroh and Johnson (1966) and Eskhol and Lunenfeld (1972) have shown clearly that LH plays an important role in the early differentiation of follicular cells.

A series of studies were performed to examine the effects of HCG (5 iu) (substituting for LH) on granulosa cell receptor content (Richards, Rao and Ireland, 1978). Oestradiol-primed hypophysectomized immature rats were injected with HCG, hFSH or HCG and hFSH. It was found that HCG given alone had little or no effect on the number of LH/HCG receptors in the granulosa cells. A single injection of hFSH, (2 µg), stimulated a small transient rise in the number of LH receptors in granulosa cells but by 48 h all the follicles had become atretic. However, when HCG was injected in combination with hFSH the number of

LH receptors per granulosa cell increased markedly by 48 h and large antral non-atretic, 'pre-ovulatory' follicles developed. The mechanisms by which HCG prevented atresia and subsequently promoted the action of a single injection of hFSH were not proposed. However, it would appear that HCG may be affecting these changes by a mechanism other than by providing testosterone, the substrate for aromatization by granulosa cells (Moon et al., 1975), since these effects were seen in rats already primed with oestradiol (Richards et al., 1978). She suggested that the small increase in LH receptors which was seen 24 h after the single injection of FSH (or 36 h after HCG) allowed HCG to act directly on the granulosa cells and to evoke responses previously associated with FSH (Ireland and Richards, 1978). However, since the LH receptor content is low in the granulosa cells of oestrogen-primed-hypophysectomized rats, it would seem more likely that the effects of HCG on granulosa cells function are mediated via a product or products, including testosterone, c'AMP and others, released as a result of HCG binding to receptors on the theca or interstitial cells (Richards et al., 1978). Specific receptors for testosterone have been identified and characterized in granulosa cells of oestrogen-primed hypophysectomized rats (Schreiber, Reid and Ross, 1976; Schreiber and Ross, 1976). However testosterone (2 mg) given in place of HCG had no effect on the ability of hFSH to stimulate an increase in the number of LH receptors or in progesterone secretion but testosterone did cause most follicles to undergo atresia (Ireland and Richards, 1978). On the other hand, the addition of testosterone or 5α dihydro-testosterone (but not oestradiol or diethylstibestrol) to culture medium containing granulosa cells from

preantral follicles resulted in dose- and time-dependent increases in progesterone production (Lucky, Schreiber, Hillier, Schulman and Ross, 1977). Other androgens have been shown to stimulate progestagen (progesterone and 20α-hydroxy-progesterone) production by cultured granulosa cells from the mature antral follicles of cyclic rats and pigs (Nimrod and Lindner, 1976; Schomberg, Stouffer and Tyrey, 1976) or granulosa cells from oestrogen-primed hypophysectomized immature rats (Nimrod, Rosenfield and Otto, 1980).

The foregoing observations prompt the following speculations. It has been suggested that the ability to secrete progestagens and to respond to androgens in vitro may represent a biochemical characteristic of the preantral granulosa cell which is retained throughout the maturation of the pre-ovulatory follicle in vivo (Hillier et al., 1980b). It has also been suggested by Richards (1978) that during the oestrous cycle, the appearance of some LH receptors in granulosa cells may allow LH, as well as FSH, to act directly on granulosa cells to promote the growth of the pre-ovulatory follicle. (The role of LH in regulating oestradiol secretion by granulosa cells at this stage was not raised). She suggested that the final stages of pre-ovulatory follicular maturation are absolutely dependent on the ability of the granulosa cells to increase their oestradiol synthesis. The actual oestradiol production would depend on the increased ability of the thecal cells to secrete aromatizable androgen in response to LH. The thecal cells therefore may hold a clue to what finally determines the ability of pre-ovulatory follicles to secrete oestradiol. Richards suggested that follicles in which the thecal cells

do not develop the ability to respond to LH may become atretic, particularly at the time of ovulation when high levels of LH are present.

These observations and speculations appear to indicate that the roles of LH and FSH in regulating the dynamics of theca-granulosa cell differentiation during early follicular development are far from being clearly resolved.

1.4 CHANGES IN STEROIDOGENESIS OCCURRING AT THE TIME OF OVULATION

The formation of steroids by the isolated pre-ovulatory follicles of rats extirpated before the endogenous LH-FSH surge on the day of pro-oestrus was extensively studied by Lindner and his co-workers (Tsafriri, Lieberman, Barnea, Bauminger and Lindner, 1973; Lieberman, Barnea, Bauminger, Tsafriri, Collins and Lindner, 1975). They found that the addition of LH *in vitro* resulted in an overall stimulation of steroidogenesis followed, 4-6 h later, by a decrease in androgen and oestrogen formation. In contrast, progesterone formation was gradually increased over and beyond this period, up to 24 h in culture. At present little is known about the site(s) through which LH has its inhibitory effects, leading to a decreased secretion of oestrogens and androgens.

In many species, the LH surge coincides with the "descending part" of the pre-ovulatory oestrogen peak, suggesting a negative effect (inhibitory?) of the presence of LH on oestrogen secretion (Cox, Mattner and Thorburn, 1971; Katz and Armstrong, 1976; Hamberger et al., 1978; Katz, Leung and Armstrong, 1979). By using the model of the PMSG-primed rat, in which ovarian steroidogenic activity is considerably

elevated (Ying and Mayer, 1969; Suzuki et al., 1978), it was demonstrated that the decline in oestrogen secretion *in vivo* is preceded by a decreased androgen biosynthesis, induced following an exogenous increase in LH (Hamberger et al., 1978). From these observations it was argued that it was the lack of suitable aromatizable substrate(s) which was the reason for the diminished oestrogen output from these ovaries (Hamberger et al., 1978). When the testosterone levels were maintained in the LH-treated rats the aromatase activity (i.e. the oestrogen secretion) was also held at its previously high level (Katz, Leung and Armstrong, 1979). These workers interpreted this result as implying that there was a protection extended by the substrate (androgen) towards the enzymes involved in its ovarian catabolism, the aromatase system. The addition of testosterone *in vitro* under similar circumstances (i.e. after the cells have been exposed to LH) also increased the formation of oestradiol by intact follicles and by theca and granulosa cells (Ahren et al., 1979).

The site(s) within the cells through which androgen synthesis is inhibited has recently been examined for the preovulatory follicles of the rat (Hamberger et al., 1978). The results demonstrate that the thecal cells are the locus for the inhibitory effect of LH on follicular androgen formation. These cells were shown to produce very low amounts of aromatizable androgens (testosterone and androstenedione) when isolated 4-8 h after the endogenous LH (FSH) surge, in spite of markedly increased production of progesterone. However at this time addition of exogenous androgen stimulated oestradiol secretion from the follicles. Conversion of 17α hydroxyprogesterone to androstenedione (see Fig. 1.8), was observed only when thecal cells

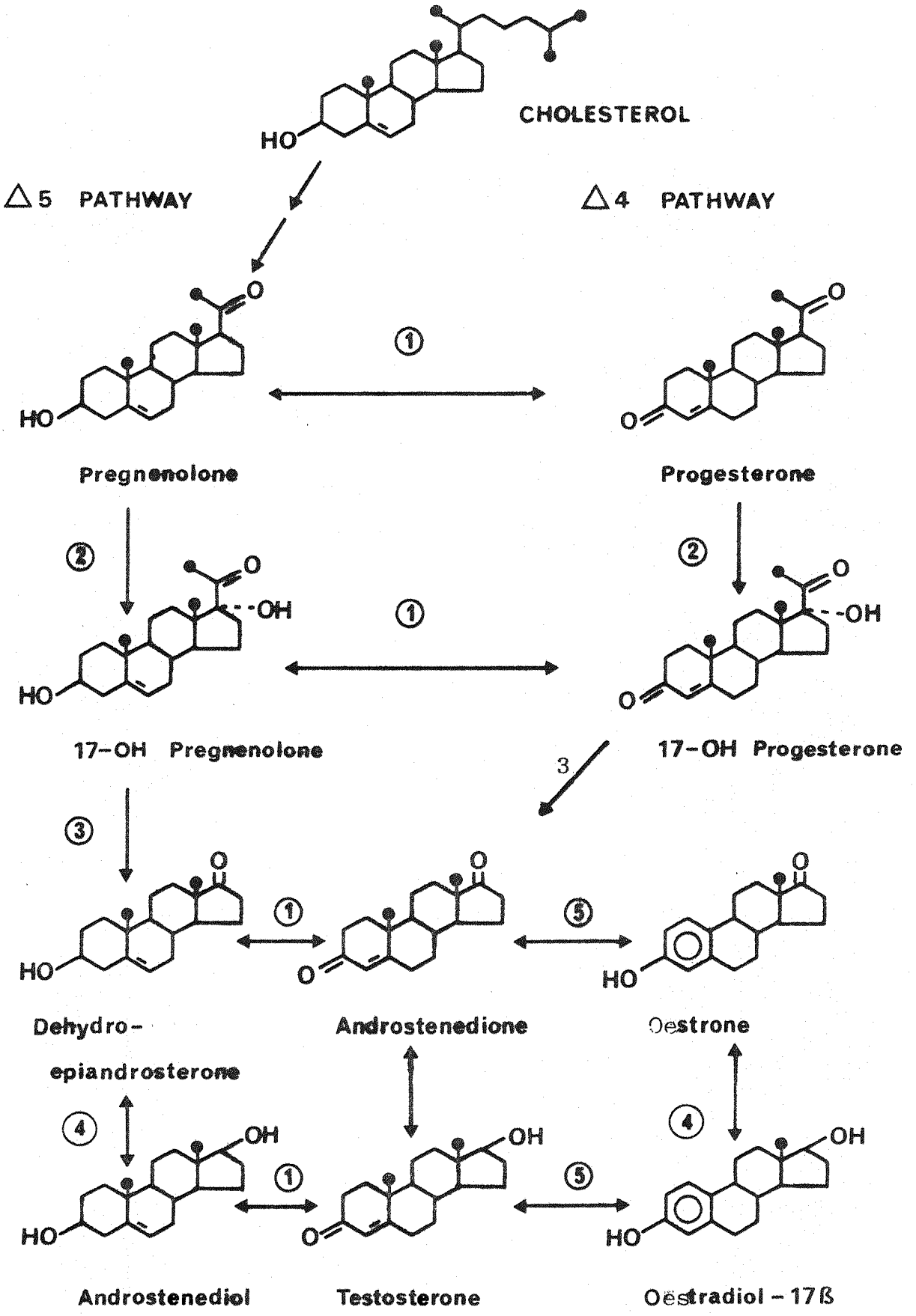


Figure 1.8 Pathways of steroid metabolism in the rat ovary. 1, Δ^5-3β-ol-dehydrogenase Δ^5-Δ^4-isomerase; 2, C_{21} steroid 17-hydroxylase; 3, C_{17-20} desmolase; 4, 17β-hydroxysteroid dehydrogenase; 5, aromatizing enzymes. (Adapted from Schulter et al. 1976).

were isolated on the morning of the day of pro-oestrus (Hamberger et al., 1978).

Whether this 'shut down' of thecal cell androgen production and/or the decline in aromatizing enzyme system (Katz and Armstrong, 1976) is the only mechanism involved in the LH-induced decline in oestradiol secretion cannot be confirmed from the preceding review. It has been suggested that LH might induce an inhibitor of the synthesis or activity of 17α-hydroxylase or 17:20 lyase; or stimulate a pre-existing population of cells short in side chain cleavage activity (Lindner et al., 1974).

However, this inhibition of the ability of the thecal cell to secrete androgen ensures that progesterone production of both the granulosa and thecal cells in response to the LH/FSH becomes unmasked (Ahren et al., 1979). This is certainly one of the components which is involved in the process of luteinization.

1.5 THE ROLE OF ATRESIA IN REGULATING THE NUMBERS OF GROWING FOLLICLES AND THE DEVELOPMENT OF PRE-OVULATORY FOLLICLES: THE ROLE OF STEROID AND PROTEIN HORMONES IN ATRESIA

So far we have been considering mainly the effect of hormones which promote follicular growth and which induce or stimulate steroid secretion. Yet ATRESIA occupies a central role in regulating the number of follicles which develop. Atresia is the term used to denote those processes during which the ovarian follicles loose their structural integrity and the oocyte degenerates. It was indicated in the previous discussion that atresia occupied a central role in regulating the pool-sizes of developing follicles, and that during the

oestrous cycle 'unsuccessful' follicles become atretic. It is known that more oocytes are lost from the mammalian ovary by atresia than are successfully shed by ovulation. For example of the 2 million oocytes estimated to be present in the human ovary at birth (Baker, 1963) only a maximum of 400 ovulate during the entire active reproductive years of a woman (Richards, 1978),

Follicles may become atretic at any stage of their development, although atresia is most common amongst the large preantral and small antral follicles. The way in which atresia occurs is not uniform but depends on the stage of development (Byskov, 1979). It has been reported that small follicles are eliminated mainly by lysis or phagocytosis of the pyknotic oocytes (Franhi and Mandl, 1962). Medium-sized follicles often become atretic without a concomitant luteinization, whereas partial luteinization characterizes the atretic large follicles. The oocyte of medium-sized follicles does not enter the maturation division during atresia whereas this is the rule in large follicles (Byskov, 1979).

The mechanisms which initiate and control atresia are not understood, but it has often been suggested that circulating hormones and/or local factors influence the rate at which follicles become atretic. Among these suggestions are: local reduction in blood supply to the follicles leading to substrate or nutrient limitation (Greenwald, 1974); or aberrant development of receptors in the theca and granulosa cells (Richards, Rao and Ireland, 1978); or the effects of locally produced or exogenous androgens (Louvet, Harman , Schreiber and Ross, 1975; Hillier and Ross, 1979). Oestrogens have a mitogenic

effect on granulosa cells in addition to their anti-atretic effect in large preantral follicles (Goldenberg, Vaitukaitas and Ross, 1972). However the incidence of atresia is increased following injections of testosterone into hypophysectomized immature rat (Payne and Runser, 1958). It has been suggested that androgens may be implicated under some circumstances with the induction of atresia. HCG injected into hypophysectomized immature female rats at doses between 0.03 to 0.3 m i.u. caused a reduction in ovarian weight, which was attributed to a marked increase in the incidence of atresia (Louvet et al., 1975). This HCG-induced atresia could be inhibited (reversed) by treating the rats with anti-androgens (Louvet et al., 1975). It can be seen from the preceding discussions that the growth of the follicles in hypophysectomized rats is stimulated by oestrogens given concommitantly with gonadotrophins. Furthermore it was argued that the local production of oestrogens within the follicle would have the same effect. Thus, the anti-atretic effect observed 24 h after injecting PMSG into intact 21-day old mice (Peters et al., 1975) might be the result of stimulated oestrogen production. In rats also, injecting PMSG prevents atresia and maintains follicular growth (Welschen, 1973). It has been suggested that FSH stimulates a renewed growth of the developing large antral follicles which are held in a 'reserve' pool (Greenwald, 1973). The 'reserve' follicles - at least in the cycling hamster - consist of an unidentified population of preantral follicles with four to five layers of granulosa cells, which normally require 8 to 12 days before developing into antral follicles (Greenwald, 1979). It has been suggested as

an alternative that the action of FSH is not to restart the growth of resting preantral follicles but rather to prevent granulosa cells degenerating and to support continued follicular growth (Peters et al., 1975). This alternative hypothesis is supported by results of experiments in which PMSG injected into immature mice resulted in a decrease in the rate of pyknosis without concommittantly increasing the rate of cell multiplication (Pedersen, 1970). It has also been suggested by Peters (1979) that PMSG acts by preventing granulosa cells from becoming pyknotic; by eliminating those pyknotic cells that are present in the granulosa cell layers via phagocytosis; and by stimulating mitosis among the granulosa cells. However, it seems unlikely that the major effect of PMSG is to prevent atresia, but it must also support the continued growth of 'rescued' follicles. With higher doses of PMSG, superovulation of 60 ova can be obtained in prepubertal rats, mice and cycling hamsters (Greenwald, 1979). These numbers cannot be accounted for solely by a reduction in atresia but, he argues, probably represents additional recruitment of smaller preantral follicles by accelerated growth. Thus, the effects of PMSG are dose dependent; smaller doses prevent some follicles from undergoing atresia, and larger doses, in addition (to preventing atresia), increase the pool of follicles developing to the antral stage.

The 'atresia-preventing' action of FSH on granulosa cells has also been demonstrated in tissue culture (McNatty, Hunter, McNeilly and Sawers, 1975). Human granulosa cells remained viable in serum devoid of LH. However, when cultured in serum devoid of FSH, only 8% of the granulosa cells remained after

10 days, while daily addition of FSH to the culture medium maintained the granulosa cells at numbers comparable to the controls (Peters, 1976). Thus, also in culture, growth of granulosa cells is maintained and atresia is prevented when FSH is present.

Our limited knowledge of the physiology of atresia is in part due to the fact that at the present time we cannot distinguish on morphological criteria, between 'healthy looking' follicles which are destined to undergo atresia and 'healthy' follicles which will complete their development with ovulation (Byskov, 1979).

However, recent studies using spontaneously ovulating rats in which ovulation had been blocked by barbiturates at pro-oestrus, have provided a model which enables one to detect the early biochemical changes which take place during atresia of pre-ovulatory follicles, before the stage when morphological signs of atresia are discernable (Uilenbroek, Woultersen and van der Schoot, 1980). In this model, the specific binding of HCG to granulosa cells was high at pro-oestrus and the next day (day 1 after ovulation had been blocked) but had decreased by day 2. The specific binding of hFSH to granulosa cells decreased gradually after pro-oestrus. Autoradiographic studies revealed that localization of HCG binding to granulosa cells and thecal cells of the pre-ovulatory follicles was still high even in those follicles which have begun to show morphological signs of atresia by day 3 (Uilenbroek et al., 1980). The ability of granulosa and thecal cells to bind HCG is associated with the large pro-oestrus follicles which are capable of producing oestrogen (Channing and Kammeram, 1973, Uilenbroek and

Richards, 1979).

The steroid producing capacity of the ovaries from these rats during the time when atresia was occurring was also studied. These studies showed that oestradiol was the major steroid accumulating in the medium from pro-oestrus follicles; this was decreased on day 1 (of barbiturate block). Androgen accumulating during these 2 days paralleled the pattern of oestradiol production (Uilenbroek et al, 1980), while the accumulation of progesterone was not different between pro-oestrus follicles and follicles obtained at day 1. The addition of testosterone to the incubation medium resulted in a significant increase in oestradiol production by follicles isolated at day 1 and day 2 (of barbiturate block). These results suggest that the low oestradiol production by the atretic follicles on day 2 may not be due to impaired aromatase enzyme activity as had previously been suggested by Moor, Hay, Dott and Cran (1978), who had studied atretic ovine follicles. Uilenbroek et al., (1980) also showed that oestradiol production was increased 10-fold by oLH from pro-oestrus follicles and 4-fold in follicles isolated at day 2. In contrast, progesterone production was increased by oLH by 100-fold in both types of follicles (Uilenbrcek et al., 1980). It was concluded from these studies that before morphological signs of atresia are apparent, follicular oestradiol production is reduced. Since pre-ovulatory follicles undergoing atresia show gonadotrophin binding and are responsive to LH with increased progesterone production, and since aromatase activity is still present, it was suggested that the enzymes involved in the conversion of progesterone to androgens are deficient in early stages of atresia of pre-ovulatory

follicles (Uilenbroek et al., 1980).

One has to remember, however, that Uilenbroek et al., (1980) did not measure LH during the time when the surge should have occurred. If this surge was present (but reduced) it might have been sufficient to cause these biochemical changes to the granulosa cells, but not large enough to cause ovulation.

1.6 SUMMARY OF THE MECHANISMS WHICH REGULATE FOLLICULAR DEVELOPMENT, ATRESIA AND THE DEVELOPMENT OF PRE-OVULATORY FOLLICLES

Follicular development is a continuous event but the rates of growth, rates of atresia and the selection of follicles for ovulation are regulated by extra- and intra-ovarian factors. The hormones required for the continued growth of the follicles to the pre-ovulatory stage appear to involve small transient increases in gonadotrophins, leading to increased synthesis of follicular androgens and oestrogens. It appears to be oestradiol which enhances the responsiveness of follicular granulosa cells to basal concentrations of gonadotrophins and thus enhances the responsiveness of the follicular granulosa-cell-LH-receptor. Furthermore, it is the continuing elevated production of oestradiol from growing pre-ovulatory follicles which ultimately stimulates the LH surge (Legan, Coon and Karsh, 1975). Thus oestradiol synchronizes the development of the pre-ovulatory follicles with the LH surge. Oestradiol and androgen levels fall with the 'onset' of the gonadotrophin surge.

However, recent progress in studies on the development of ovarian follicles which has been reviewed here has helped us to understand the complex cellular interactions which exist

within the specialized micro-environment of each follicle. We have begun to gain insight into how steroid and pituitary hormones interact to cause ovarian cell differentiation. We have begun to understand the rate limiting role of androgen production which is revealed following the endogenous gonadotrophin surge. Finally we have begun to understand what events may be involved in atresia. Although the significance of atresia in ovarian follicular development is well documented, many of the processes involved in selecting for ovulation a few oocytes from thousands available remain a mystery.

1.7 THE AIM OF THE INVESTIGATIONS REPORTED IN THIS THESIS

Most of the previous research concerning the effect of HCG or LH on steroid production by the ovary has been carried out on isolated granulosa or thecal cells. However, since aromatizable androgens are produced not only by the follicle in which they are subsequently aromatized, but also by thecal tissue of small follicles and by interstitial tissue, we have used whole ovaries for some of our studies. We postulated that interactions between follicles of different sizes might influence the steroidogenic capacity of the organ as a whole. We speculated that the rate of oestradiol production in the pre-ovulatory ovary might be limited by the supply of testosterone. We investigated the effects of trophic hormones acting on whole ovaries to stimulate oestradiol secretion. We have attempted to mimic the situation which occurs *in vivo* during follicular maturation and the LH surge. We also speculated that by using inhibitors of steroidogenesis, we might find that an inhibition of, for example, protein synthesis might

result in a parallel inhibition of steroidogenesis.

Secondly, we postulated that androgens which are known to be present in large amounts in the ovaries of immature rats (namely, 5α-androstane - 3α,17β-diol, and 5α-androstane-3β-17β-diol) might influence the ability of ovaries to secrete oestradiol and progesterone. For this, we used isolated granulosa cells since we wished to localize the site of action of these steroids.

The experimental model we used was the immature female rat primed with 5 iu PMSG at 26 days of age, 48 h before the experiment. The main advantage of this model is that pre-ovulatory follicular development is synchronized so that elevated levels of steroids produced by ovaries following PMSG treatment permit experimentally induced changes to be followed more accurately. An additional advantage of this model is the lack of the endogenous gonadotrophin surge, which was important for the study of the intraovarian gonadotrophin - steroid interactions which are the major factors in follicular development and function.

We used HCG instead of LH for most parts of the project because of the biological similarities of the two gonadotrophins (HCG binds to the same receptors as LH), although we did compare the biological activities of our two hormone preparations. Also we used PMSG as a substitute for FSH because PMSG has FSH-like activity and also has a longer half-life. We also compared the biological activities of our PMSG and FSH preparations. However, in later studies with granulosa cells and Leydig cells we used purified ovine FSH and ovine LH kindly donated by Dr. A.F. Parlow of NIAMDD.

The experimental component of this thesis begins with a brief introduction and a description of the methods, followed by sections in which each topic which has been investigated is introduced, reported and discussed separately. Finally, a summary of the data is compared with results of other workers in the field, and an overall hypothesis is proposed to explain and to integrate our results with those of others.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

The animals used for this research were either immature female Wistar rats aged 25-28 days, weighing 45-60 g, or adult rats of the same strain. All animals were bred in the University's animal house. The immature rats were weaned on day 21. They were allowed food and water ad libitum. The animals were housed with a maximum of 4 per cage and placed on a lighting regime of 14 h light/10 h dark per 24 h. The immature animals were killed by cervical dislocation while the adult rats were anaesthetised with sodium pentobarbitone i.p. Blood was collected by cardiac puncture into lightly heparinized syringes. The ovaries were removed for incubation or histology. The uteri were removed, cleared of connective tissue and weighed when appropriate.

2.2 HORMONE TREATMENT

The gonadotrophic hormone preparations used were highly purified ovine FSH (Batch No. NIAMDD - OFSH ≠ 13, with a reported biopotency of 15 u/mg) and ovine LH (Batch No. NIAMDD - LH ≠ 21, with a reported biopotency of 2,5 u/mg). The hormones were gifts from the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD). The hormones were dissolved in Kreb's ringer bicarbonate (KRB) containing 0.5% Bovine Serum albumin (BSA) to the required working concentration. Pregnant mares' serum gonadotrophin [(PMSG), batch No. 87C-04831, containing 1970 iu/mg protein] was supplied by Sigma Corporation

Ltd. 'Pregnyl', Human Chorionic Gonadotrophin [(HCG) batch No. 6299 containing 3250 iu/mg protein] was obtained from Organon Ltd. The latter hormones were usually made up in saline (9 g NaCl/l) to the required working concentration.

The immature female rats were injected subcutaneously with 5 iu/ml PMSG and the control animals with carrier volume of saline.

The comparative biological activities of these hormonal preparations were subsequently tested (see Chapter 4).

2.3 INCUBATION PROCEDURES

The technique used to incubate the ovaries *in vitro* was a modification of that of Fainstat (1968). Immediately after killing the animals, the ovaries were dissected free of all connective tissue and placed in incubating dishes containing 1 ml Kreb's Ringer Bicarbonate buffer (KRB) pH 7.4 supplemented with 1.1 mM glutamine and 0.2% glucose. The incubation dishes containing ovarian tissues were placed in an incubation chamber and gassed with 95% O_2 : 5% CO_2.

The initial problem encountered in this incubation technique was extensive evaporation when the chamber was gassed continuously. The evaporation was greatly reduced by gassing the incubation intermittently, and by saturating the gas with water vapour before it entered the incubation chamber.

2.4 HISTOLOGICAL PROCEDURE

Immature female rats were killed 0 (control), 24, 48 and 72 h after PMSG (5 iu) or 0.1 ml saline had been injected on day 25. Ovaries were removed, cleaned of connective tissue

and fixed in Bouin's solution. They were later dehydrated in graded solutions of alcohol. After embedding in paraffin wax, serial sections (10 µm) were cut, mounted on slides, and stained with haematoxylin and eosin.

The histology of the ovaries from each group of animals was scrutinized for the development of pre-ovulatory follicles, corpora lutea and the presence of atretic granulosa cells in the antral follicles.

2.5 CHEMICALS

The chemicals used in this research and their commercial sources are listed below.

i. Reagents used to prepare the buffers

Di-sodium hydrogen orthophosphate	British Drug Houses
Potassium dihydrogen orthophosphate	British Drug Houses
Sodium chloride	British Drug Houses
Potassium chloride	British Drug Houses
Magnesium sulphate $-7H_2O$	British Drug Houses
Calcium chloride	British Drug Houses
Sodium bicarbonate	British Drug Houses

ii. Reagents for celite chromatography

Ethanediol	British Drug Houses
Diatamaceous Earth (Grade III)	Sigma Chemical Co.
Iso-octane (2, 2, 4,-Trimethyl pentane)	British Drug Houses
Benzene	Koch-Light Labs.
Chloroform	Koch-Light Labs.
Methanol	Koch-Light Labs.
Ethyl Acetate	Koch-Light Labs.

iii. Stains and mounting medium used for histology

Erlich's Haematoxylin	Hopkin and Williams
DPX (mounting medium)	Hopkin and Williams

iv. Radiochemicals

All radiochemicals were obtained from Radiochemical Centre, Amersham, Buckinghamshire, England.

(1, 2, 6, 7n - ^{3}H) Testosterone	SA 80-90 Ci/mmol.
(2, 4, 6, 7n - ^{3}H) Oestradiol	SA 80-90 Ci/mmol.
(1, 2, 6, 7n - ^{3}H) Progesterone	SA 80-110 Ci/mmol.
(4 - ^{14}C) Testosterone	SA 53.3-59.4 nCi/mmole.
(4 - ^{14}C) Progesterone	SA 60.7 mCi/mmole.
DL - (4, 5 - ^{3}H) Leucine	SA 44 Ci/mmol.

v. Unlabelled Steroids

Testosterone (T)	Sigma Chemical Co.
Dihydrotestoterone (DHT)	Sigma Chemical Co.
Progesterone (P)	Sigma Chemical Co.
17 α-hydroxyprogesterone (17αOHP)	Sigma Chemical Co.
Dihydroepiandrosterone (DHEA)	Sigma Chemical Co.
Pregnenolone (Δ^{5}-P)	Sigma Chemical Co.
Oestradiol - 17 β (E_2 - 17β)	Organon Ltd.
Oestradiol - 17 α (E_2 - 17α)	Steraloids Inc.
5α-androstan-3α-17β-diol (αβ)	Sigma Chemical Co.
5α-androstan-3β-17β-diol (ββ)	Sigma Chemical Co.
Androstenedione (A_1)	Sigma Chemical Co.
Oestrone (E_1)	Sigma Chemical Co.
Oestriol (E_3)	Sigma Chemical Co.
5α-androstanedione (5α-A)	Sigma Chemical Co.

vi. Inhibitors of protein synthesis

Puromycin	Sigma Chemical Co.
Cycloheximide	Sigma Chemical Co.

vii Other reagents and chemicals

Leucine	British Drug Houses
Freund's adjuvant	Miles Laboratories Inc.
Sodium pentobarbitone ('Sagatal')	May and Baker Ltd.
Urethane	Sigma Chemical Co.
Heparin	Boots Co. Ltd.
Acetone	Koch-Light Laboratories
Diethyl ether (puriss, anhydrous)	Koch-Light Laboratories
Ethanol	J. Burroughs Ltd.
Glacial acetic acid	Koch-Light Laboratories
Trichloro-acetic acid (TCA)	Koch-Light Laboratories
Potassium hydroxide (KOH)	British Drug Houses
Sodium carbonate	British Drug Houses
Copper sulphate	British Drug Houses
Sodium potassium tartrate	British Drug Houses
Sodium deoxycholate	British Drug Houses
Folins Ciocalteu's phenol reagent	British Drug Houses
Bovine serum albumin	British Drug Houses
Collagenase (Type 1)	Sigma Chemical Co.
Charcoal Norit A.	British Drug Houses
D-Glucose	British Drug Houses
Gelatin	Sigma Chemical Co.
Sodium azide	Sigma Chemical Co.
Dextran T 70	Pharmacia Fine Chemicals
Medium 199	DIFCO Laboratories

2.6 PREPARATION OF BUFFERS

i. Phosphate-gelatine-buffer, pH 7.4 (50 mM $Na_2H\ PO_4$ and 50 mM $KH_2\ PO_4$)

a. Stock solutions were made up containing 0.01 g/l sodium azide.

b. These were titrated against each other to reach pH 7.4.

c. 1 g of gelatin was dissolved in 100 ml phosphate buffer pH 7.4 and made up to 1 litre.

ii. Dextran Charcoal Preparation

To 200 ml of gelatine-phosphate-buffer pH 7.4 was added 500 mg charcoal and 50 mg dextran T 70. This was put into a conical flask containing a magnetic stirrer and stirred for about 1 hour before being stored at 4°C.

iii. Kreb's Ringer Bicarbonate

	g/l
NaCl	5.50
KCl	0.35
$MgSO_4 . 7H_2O$	0.11
$CaCl_2$*	0.28*
$KH_2\ PO_4$	0.16
$NaHCO_3$	2.10

*Calcium chloride ($CaCl_2$) was separately dissolved in 100 ml of distilled water before added to 800 ml solution containing the above listed salts. It was then made up to 1 litre. The buffer was gassed before use with 95% O_2 : 5% CO_2 for approximately 20 minutes until pH 7.4 was obtained. 0.2% D-glucose and 1.1 mM glutamine were added prior to use.

2.7 CHOICE OF INCUBATION MEDIUM

Incubations were initially set up in medium 199. Subsequently, we decided to culture our ovarian tissues in an alternative medium, Kreb's Ringer bicarbonate supplemented with glucose and glutamine. This medium offers the advantage of being less expensive and also the ionic composition can be changed readily. Dufau and Catt (1975) and Ben-Or and Broza (1970) successfully used this latter medium in their studies of trophic hormone action on testes and ovaries.

Our preliminary studies on culturing PMSG-primed or saline treated control rat ovaries in medium 199 or Kreb's Ringer bicarbonate buffer containing glucose and glutamine showed no significant difference in the amounts of the steroids accumulating in each of these media.

2.8 LIQUID SCINTILLATION FLUIDS

The liquid scintillation fluids used throughout were Tritoscint or Toluene-Butyl PBD consisting of the following:

i. Tritoscint

Xylene	2 litres	Koch-Light Laboratories
PPO	12 g	G & G Chemicals
Dimethyl POPOP	0.9 g	G & G Chemicals
Synperonic NXP non ionic detergent	1 litre	Cargo Fleet Company, Stockton on Tees

ii. Toluene-Butyl PBD

Toluene (Sulphur free)	2.5 l	Koch-Light Laboratories
Butyl PBD	20 g	G & G Chemicals

2.9 ANTIGENS

a)	Oestradiol-6-carboxymethyl-oxime-bovine serum albumin (BSA)	Steraloids Inc.
b)	Progesterone-11-carboxymethyl-oxime-BSA	Steraloids Inc.
c)	Testosterone-11-carboxymethyl-oxime-BSA	Steraloids Inc.

2.10 CELITE CHROMATOGRAPHY

Celite chromatography similar to that previously described by Barberia and Thorneycroft (1974), was set up to separate steroids in plasma and incubation medium

Preliminary studies on the effect of heating or not heating the celite prior to making the slurry showed that there was no effect on the elution profile of the steroids. A slurry of approximately 2:1 ethanediol:diatamaceous earth (celite) (V/V) was made up and poured into pasteur pipette containing a glass fibre plug. The columns were allowed to settle overnight and a column of approximately 5 cm was obtained. Each column was washed with 2 ml iso-octane prior to adding the steroids. The steroids were eluted as shown on Table 2.1. The fractions in which the steroids were eluted had earlier been established by adding a pair of labelled steroids e.g. ^{3}H-DHT and ^{14}C-T to the column and eluting with varying volume of solvents.

Table 2.1 Elution of various steroid from celite column with ethylene glycol as stationary phase.

Fraction No.	Volume of Eluate (ml)	Eluate	Steroid Eluted
1	1.5	iso-octane	Progesterone Pregnenolone
2	2.5	iso-octane	Androstenedione
3	3.5	95% iso-octane 5% Benzene	Dihydrotestosterone
4	3.5	60% iso-octane 40% Benzene	Testosterone 17α OH-Progesterone
5	3.5	40% iso-octane 60% Benzene	5α-Androstanediol
6	3.5	15% Ethyl acetate 85% iso-octane	Oestrone
7	3.5	40% Ethyl acetate 60% iso-octane	Oestradiol

2.11 PREPARATION OF GRANULOSA CELLS

Twenty-six-day rats were injected subcutaneously (s.c.) with 5 iu PMSG at 10.00 am. The animals were killed between 10.00 and 12.00 h on day 28. The ovaries were removed, dissected free from the connective tissue, placed in ice-chilled Kreb's solution and the large follicles were isolated under a stereomicroscope. Granulosa cells were isolated as previously described by Hamberger, Hillensjo and Ahren (1978) and by Hillier, van den Boogaard, Reichert and van Hall (1980). In brief, granulosa cells were expressed from individual follicles (0.8-1.0 mm diameter) and the remaining fragment of follicular tissue was discarded. The granulosa cells were sedimented by centrifugation at room temperature (5 min at 700 g) and re-suspended in fresh chilled incubation medium. The cell

concentration was determined using a haemocytometer and the cells diluted and aliquoted out so that the incubations were carried but using approximately 1 x 10^5 cells/ml KRB. The viability of the cells was evaluated by diluting one aliquot with 0.4% Trypan blue. The percentage of cells which excluded the dye was always greater than 50%.

2.12 PREPARATION OF LEYDIG CELLS

Leydig cells were separated from other testicular cells by the method described by Janszen, Cooke, Van Driel and Van der Molen (1976). Adult rats were anaesthetised with sodium pentobarbitone i.p. and the testes quickly removed and decapsulated. The testes were placed in a flask containing 7 ml KRB supplemented with 0.2% glucose (KRBG) at pH 7.4, and 1 mg/ml collagenase and 1 mg/ml BSA. Incubation for 20 min at 37^{o}C was carried out under an atmosphere of 95% O_2 : 5% CO_2 in a shaking water bath set at 75 oscillations/min. After incubation, 15 ml of 0.9% NaCl were added to the flask, which was shaken and allowed to stand at room temperature for 10 mins. The supernatant was filtered through nylon gauze, centrifuged in a bench centrifuge (5 min at 700 g) at room temperature. The supernatant was discarded while the cells were resuspended in 15 ml KRBG. An aliquot of the cell suspension was counted in a haemocytometer to determine the cell density. Incubations were finally carried out using approximately 0.4 x 10^5 cells/ml.

2.13 STATISTICAL ANALYSIS

Statistical analysis was performed using paired or unpaired student's t-tests where appropriate. Linear regression and correlation coefficients were calculated using a programmable Hewlett-Packard digital calculator. Data on individual experimental groups were also analysed using one way analysis of variance; values of $P < 0.05$ were taken to be significant. Other statistical analyses used are described in the appropriate legends. Experimental data are presented as mean values $\pm$ SEM.

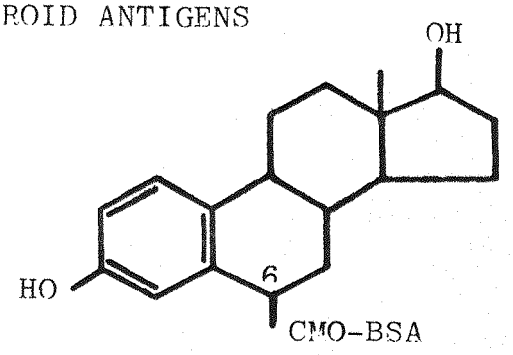
CHAPTER 3

RADIOIMMUNOASSAY TECHNIQUES

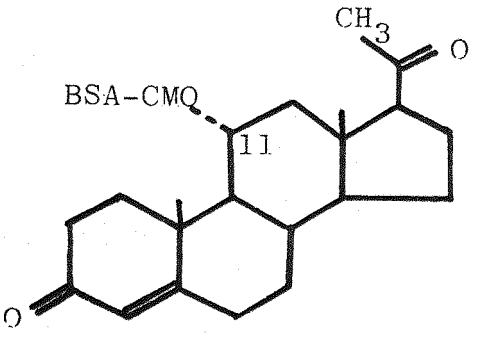
3.1 INTRODUCTION

Radioimmunoassay (RIA), like competitive binding analysis, is an example of saturation analysis which depends on the reversible binding of the compound being assayed with specific proteins. Steroids, unlike protein hormones, are not antigenic, so it is necessary first to couple the steroid covalently to a large protein such as bovine serum albumin (BSA). In this way, when the combined steroid-protein conjugate (the antigen) is injected into rabbits, antibodies against the hapten group on the antigen are formed. Under these circumstances, the steroid residues appear to act as the hapten groups. These antibodies have a high binding affinity for the original steroid molecule used. The specificity of the antibodies formed depend very much on the site through which the albumin is coupled to the steroid molecule. Coupling is usually done at sites far remote from existing functional groups on the steroid so that the antigenic information of the steroid is not masked, since even small variations in the nature or orientation of a substitute in the functional groups at position 3, 17 or 20 could result in major differences in steroid specificity and potency (Lindner and Bauminger, 1974). The antigens we used are shown in Fig. 3.1. All three steroids were coupled to bovine serum albumin through carboxymethyloxime (CMO) at either position 11 or 6.

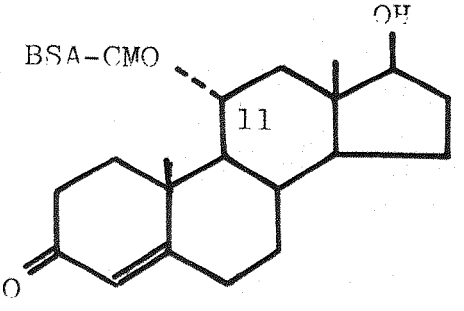
FIGURE 3.1 STRUCTURE OF STEROID ANTIGENS



oestradiol-17β antigen



progesterone antigen



testosterone antigen

The steroid antigens oestradiol-17β-6-CMO-BSA, Progesterone-11-CMO-BSA and Testosterone-11-CMO-BSA were used to raise antisera for the radioimmunoassays described in the text. The BSA has been linked to the steroid residues through sites on the steroids distant from the functional groups on the steroids.

The use of RIA techniques has made it possible to eliminate preliminary purification of the steroids by chromatography provided that the antibodies which are used are specific to the steroid. Therefore a larger number of samples may be dealt with at any one time. RIA for oestradiol, testosterone and progesterone have all been set up and characterized in our department. These assays have made it possible to measure directly the plasma steroid levels in immature and adult rats as well as the steroid concentrations in incubation media from experiments in vitro.

3.2 PRODUCTION OF ANTISERA AND CHARACTERIZATION OF ANTIBODIES

Setting up a radioimmunoassay

Antisera were raised in rabbits (New Zealand White) to one of the following antigens:

1. Oestradiol-6-carboxymethyl-oxime-bovine serum albumin.
2. Progesterone-11-carboxymethyl-oxime-bovine serum albumin.
3. Testosterone-11-carboxymethyl-oxime-bovine serum albumin.

The rabbits were injected intra-dermally with 1.0-2.0 mg of the conjugate in 50:50 mixture of saline in complete Freund's adjuvant. They received two supplementary intra-muscular injections 6 and 8 weeks later. The antiserum titre was estimated at 10 and 12 weeks before the animals were killed by exsanguination, under urethane anaesthesia, through an aortic cannula. The antisera were stored in 500 µl aliquots at -20°C until used.

The assay procedure was similar for all 3 steroids except that the antisera were used at different dilutions. The titre of each antisera was found by incubating the tritiated hapten

(∿10,000 dpm/ml) with dilutions of the antisera from 1:10 to 1:100,000 for 3h at 37°C. The free and the bound ^{3}H-steroid were separated using dextran coated charcoal, at 4°C, and after counting the radioactivity in the bound fraction, the dilution of the antiserum at which the label was 50% bound was calculated. These dilutions were: for oestradiol 1:22,000; progesterone and testosterone 1:8,000.

3.3 METHOD FOR EXTRACTING STEROIDS FROM PLASMA AND MEDIUM SAMPLES

100 µl aliquots of plasma or 200 µl incubation medium containing the secreted steroids, were extracted in 12 x 100 mm glass test tubes with 2 ml ether. 100 µl ethanol were added to each plasma sample prior to extracting the steroid to denature the binding proteins present. Each sample was vortexed for 1 min, the aqueous phase frozen on cardice (solid CO_2) and the supernatant decanted into 12 x 75 mm glass test tubes and evaporated overnight in the fume cupboard at room temperature.

3.4 METHODS USED FOR RADIOIMMUNOASSAY

Standard curves were constructed for each steroid over the following ranges:

Oestradiol: 2.5×10^{-14} moles to 5×10^{-12} moles per tube

Progesterone: 5×10^{-14} moles to 5×10^{-12} moles per tube

Testosterone: 5×10^{-14} moles to 2.5×10^{-11} moles per tube.

The purity of these standards were routinely checked by gas liquid chromotography by Dr. D. Corina, and the steroids were found to give only one retention peak.

The assay procedures were performed as follows:

Stock antisera was diluted with 50 mM phosphate buffer pH 7.4 containing 0.1% gelatine and 0.01% sodium azide to the dilution binding 60-70% of the added tritiated steroid, to which the antibody had been raised. The diluted antisera were incubated at 4°C overnight with the homologous tritiated steroid (^3H - E_2; ^3H - P or ^3H - T) so that each tube finally contained approximately 20,000 dpm.

500 µl of the tritiated binding solution were added to each assay tube, the sample mixed and incubated at 37°C for 1½ hours, vortexed again and left for a further 1½ hours at 37°C. The samples were then chilled on ice for 10 minutes. Free and bound steroid were separated using 500 µl dextran-coated charcoal. The tubes were vortexed, left to stand on ice for 10 minutes, centrifuged at 4°C at 2,000 rpm on an MSE mistral 2L centrifuge. 500 µl of the supernatant were pipetted out into scintillation vials, and 500 µl distilled water and 10 ml tritoscint added. The radioactivity was counted for 4 minutes in a Philip Liquid Scintillation Counter with a counting efficiency of 55% and which gave automatic quench correction and printed the results as corrected dpm.

In all assays, pool samples were incubated as well as reagent and assay blanks. This enabled the precision of the assay, intra-and inter-assay variation, as well as the sensisivity of the assay, to be established.

3.5 ANALYSIS OF ASSAY DATA

Analysis of the assay data was performed using a Hewlett-Packard digital calculator programmed to perform a logit-

transformation of the dpm, and to estimate the amount of steroid in the unknown samples from a calculated line of closest fit to the standard curve. (This programme was prepared for us by Dr. I. Giles, to whom we are grateful).

Any sample which fell within 9.89% of either end of the line was considered either non detectable since the logit-transformation deviates from linearity beyond these limits.

The coefficient of regression, the slope and the intercept value on the Y axis were also calculated. A typical standard curve for one of the steroids is shown in Fig. 3.2.

3.6 ASSESSMENT OF ASSAY SENSITIVITY, REPRODUCIBILITY AND SPECIFICITY

Minimum sensitivity, i.e. the least detectable amount measured in the assay as obtained from the standard curve was 2.5×10^{-14} moles for oestradiol. Progesterone and testosterone both had minimum sensitivity of 5×10^{-14} moles per tube.

The reproducibility of the assay was determined from the pool samples used in each assay and from these results the inter-assay coefficients of variation (CV) of the assay was found by using the following formula:

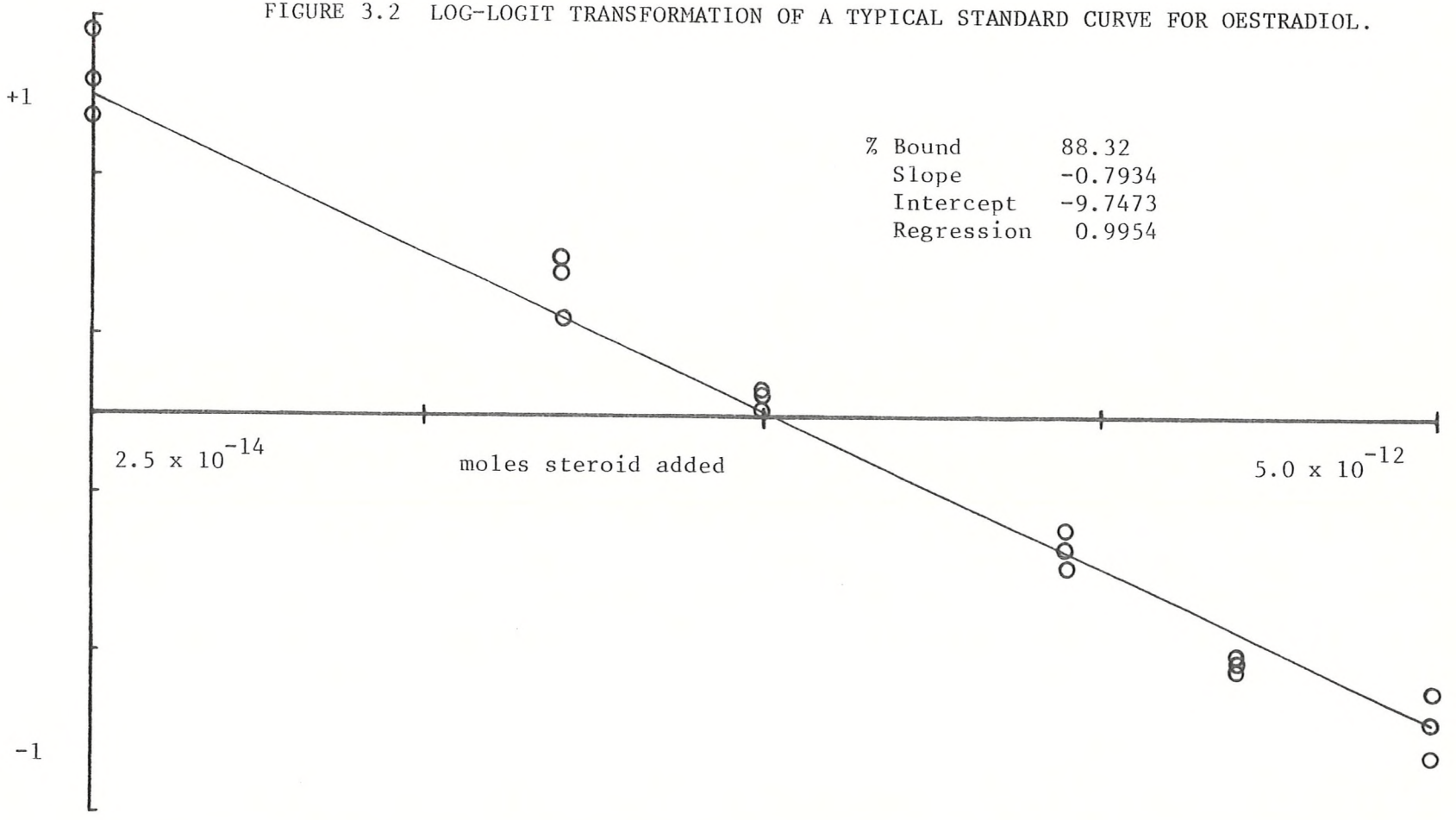
$$CV = \frac{SD}{\bar{x}} \times 100$$

where $\bar{x}$ = the mean value of the steroid pool samples from different assays and

SD = standard deviation of the mean of the pool samples.

Intra-assay variation was also determined in the same way where $\bar{x}$ is the mean value of amount of steroid present in tubes containing the same amounts of added steroid. The inter-assay

FIGURE 3.2 LOG-LOGIT TRANSFORMATION OF A TYPICAL STANDARD CURVE FOR OESTRADIOL.



Antiserum raised against E_2-6-CMO-BSA at a final dilution of 1:22,000 was incubated with ^{3}H-oestradiol and the radioactivity displaced with cold steroid, over a range between 2.5×10^{-14} moles and 5×10^{-12} moles; standards were run in triplicate. The details of the method are in the text.

coefficients of variation of oestradiol assay were 12.2%, 11.9% and 14.5% for mean KRB pool samples of 8.5×10^{-13} moles/ml, 2.04×10^{-12} moles/ml and 4.15×10^{-12} moles/ml respectively while the intra-assay coefficients of variation were 7.4%, 6.1% and 7.9% respectively for the same pool samples. For progesterone inter-assay coefficients of variation of the three pool samples of 2.1×10^{-12} mole/ml, 1.96×10^{-12} mole/ml and 3.4×10^{-12} mole/ml were 12.7%, 11.1% and 12.9% respectively, while the intra-assay coefficients of variation were 7.0%, 6.6% and 5.9% respectively. For the testosterone assay, the inter-assay coefficients of variation were 12.3%, 11.2% and 12.9% for mean pool samples of 1.01×10^{-12} moles/ml, 1.86×10^{-12} moles/ml and 2.67×10^{-12} moles/ml while intra-assay coefficients of variation were 7.0%, 6.1% and 6.5% respectively (n = 15 in each instance).

Medium blanks were always below the minimum sensitivity of the assays and the reagent blank was taken into account in the programme for the logit-transformation on the Hewlett-Packard digital calculator.

The recovery of added steroids from the medium was estimated by adding a known amount of steroid (1×10^{-7} M) in triplicates and measuring in the same way as the unknown samples. From the standard curve, the concentration of the steroids were determined and expressed as the % of the added steroid. The recovery in the assays were 95.4% $\pm$ 2.8. for oestradiol, 94.5% $\pm$ 1.9% for testosterone and 97.9% $\pm$ 3.7% for progesterone; short fall in recovery has not been corrected for since it seemed to be consistent at three different doses (50, 75 and 100 µl) of the assayed steroids.

Recoveries during the extraction procedure were checked by adding the homologous tritiated steroid to the plasma prior to ether extraction. These were found to be 95.4 ± 2.8% for oestradiol, 92.9 ± 1.95% for testosterone and 89.9 ± 2.4% for progesterone, while recoveries after celite chromatography were 84.2 ± 3.5%, 87.9 ± 2.8% and 88.9 ± 1.7% for oestradiol, testosterone and progesterone respectively (n = 15 in each instance).

The cross reactivity of the antisera were evaluated at the level at which the added antigen gave a 50% displacement of the tritiated label, 100% being the % bound in the absence of added cold hapten (Abraham, 1969). At this level for oestradiol (E_2-17β) antiserum, oestradiol-17α (E_2-17α), oestrone (E_1) and oestriol (E_3) caused significant displacements being 50.0%, 24.9% and 9.1% respectively of that caused by E_2-17β. Other steroids tested (Testosterone, androstenedione, progesterone and 11α-OH-progesterone) caused less than 1% displacement.

At the 50% level for the testosterone antiserum, only dihydrotestosterone (DHT) caused any significant displacement, being 50% of that caused by testosterone. Androstenediol, androstenedione, androstanediols, dehydroepiandrosterone (DHEA), oestradiol-17β, oestrone, progesterone and 11α-OH progesterone caused less than 1% displacement.

At the 50% level of the progesterone antiserum, 11α-OH-progesterone and 17α-OH-progesterone gave 95% and 35% cross reactivity respectively of that caused by progesterone. All the androgens and oestrogens tested caused less than 1% displacement. These results together with the other data relating to the validation of the assays are summarized in Table 3.1.

All samples for one experiment, with their controls, were always assayed together, obviating the necessity to correct for inter-assay variations.

Table 3.1 Summary of antisera characterization

The assays used antisera raised in our department, and a logit/log transformation of the standard curve.

Oestradiol Assay	
Minimum sensitivity	2.5×10^{-14} moles/tube
Intra-assay coefficient of variation (CV)	6.1% - 7.9%
Inter-assay coefficient of variation (CV)	11.9% - 14.5%
Medium blank value	$< 1.8 \times 10^{-14}$ moles

Cross Reaction at the 50% Displacement Level	
Oestradiol-17β	100%
Oestradiol-17α	50%
Oestrone (E_1)	24.9%
Oestriol (E_3)	9.1%
Other steroids	< 1%

Progesterone Assay	
Minimum sensitivity	5×10^{-14} moles/tube
Intra-assay coefficient of variation (CV)	5.9% - 7.0%
Inter-assay coefficient of variation (CV)	11.1% - 12,9%
Medium blank value	2.0×10^{-14} moles

Table 3.1 (Cont)

Cross Reaction at 50% the Displacement Level	
Progesterone	100%
11α-OH-progesterone	95%
17α-OH-progesterone	35.5%
Other steroids	< 1%

Testosterone Assay	
Minimum sensitivity	5.0×10^{-14} moles/tube
Intra-assay coefficient of variation (CV)	6.1% - 7.0%
Inter-assay coefficient of variation (CV)	11.2% - 12.9%
Medium blank value	$< 2.4 \times 10^{-14}$ moles

Cross Reaction at the 50% Displacement Level	
Testosterone	100%
Dihydrotestosterone (DHT)	50%
Other steroids	< 1%

Specificity of the assay

In order to check that the specificity of the assay was not impaired by directly measuring the steroid in the medium, a preliminary study was performed. Aliquots of the medium were extracted with ether and steroids were separated by celite chromatography. Neither the extraction step nor the subsequent chromatographic step caused any significant reduction in the levels of oestradiol, testosterone and progesterone otherwise obtained in the unextracted sample. These results are compared in Table 3.2. Similarly, there was no significant difference in the levels of oestradiol, testosterone or progesterone in

the ethereal extracts of plasma assayed directly or the ethereal extracts which had been further subjected to celite chromatography (Table 3.3). These results may indicate that interfering steroids were not present in the medium or in plasma in significant amounts. Subsequent determinations of these steroids were routinely carried out on unextracted samples of medium or on ethereal extract of plasma, since the antisera had been shown to be highly specific.

Table 3.2 Effect of sample pre-purification on steroid estimations on media collected from PMSG-primed immature rat ovaries after a 4 h incubation. Results are expressed as means ± SEM of 4 incubations.

Treatment	Oestradiol (pmole/ml)			Testosterone (pmole/ml)			Progesterone (pmole/ml)		
	No[a] Purification	b Extraction	c Extraction and celite chromatography	No[a] Purification	b Extraction	c Extraction and celite chromatography	No[a] Purification	b Extraction	c Extraction and celite chromatography
Control	1.20 ± 0.21	1.14 ± 0.21	1.01 ± 0.11	2.80 ± 0.21	2.61 ± 0.32	2.41 ± 0.31	1.68 ± 0.22	1.51 ± 0.21	1.49 ± 0.22
HCG	1.95 ± 0.21	1.79 ± 0.16	1.71 ± 0.22	4.84 ± 0.51	4.58 ± 0.51	4.25 ± 0.52	2.74 ± 0.31	2.44 ± 0.32	2.50 ± 0.32

The experiment was performed with ovaries harvested from 28 day old rats previously primed with PMSG (5 iu) for 48 h. The ovaries were incubated in hormone free medium (control) or in medium containing HCG (1.0 iu/ml).

[a]Assay performed directly on unextracted medium

[b]Assay performed on ethereal extract of the medium

[c]Assay performed on ethereal extract after celite chromatography. The procedure of the chromatography is as described in section 2.10.

Table 3.3 Comparison of steroid estimations obtained by radioimmunoassays applied to ethereal extracts of plasma before or after celite chromatography+.
Results are expressed as means ± SEM (n = 7).

Volume of plasma assayed (ml)	Before chromatography (pmole/tube)			After chromatography (pmole/tube)		
	Oestradiol	Testosterone	Progesterone	Oestradiol	Testosterone	Progesterone
0.05	0.10 ±0.01	1.48 ± 0.16	1.88 ± 0.09	0.09 ± 0.01	1.27 ± 0.11	1.92 ± 0.2
0.10	0.18±0.03	2.75 ± 0.31	3.07 ± 0.4	0.17 ± 0.01	2.44 ± 0.36	3.30 ± 0.22
0.15	0.26±0.04	3.66 ± 0.52	5.23 ± 0.32	0.24 ± 0.03	3.38 ± 0.14	5.44 ± 0.50
0.20	0.34±0.01	5.36 ± 0.42	6.06 ± 0.74	0.31 ± 0.02	4.52 ± 0.50	6.67 ± 0.26
0.25	0.42 ±0.03	6.74 ± 0.72	7.90 ± 0.82	0.40 ± 0.01	6.14 ± 0.58	8.4 ± 0.6
Slope*	0.007924 ± 0.000017	0.131 ± 0.007	0.151 ± 0.010	0.000732 ± 0.00001	0.118 ± 0.007	0.163 ± 0.007
Inter-cept	0.118	0.318	1.580	0.110	0.036	1.248
r	0.99	0.99	0.99	0.99	0.99	0.99

r The correlation coefficient, was calculated from the calculated line of closest fit to the data, using an analysis of least squares, and a Hewlett-Packard digital calculator.

Slope* Slopes were compared by analysis of variance using a Hewlett-Packard digital calculator. There was no significant difference between the slopes of these lines before or after celite chromatography for the 3 steroids assayed.

+ Celite chromatography was performed as described in section 2.10.

3.7 ANALYSIS OF PLASMA STEROID CONCENTRATION DURING THE FOUR-DAY OESTROUS CYCLE OF THE WISTAR RAT. A FURTHER VALIDATION OF THE RADIOIMMUNOASSAYS

The changes in the levels of the ovarian steroids in the blood during the oestrous cycles of the rat are well documented (Brown-Grant, Exley and Naftolin, 1970; Barraclough, Collin, Massa and Martini, 1971; Dupon and Kim, 1973 and Smith, Freeman and Neill, 1975). Dupon and Kim (1973) demonstrated that the secretion profiles of oestradiol and testosterone are similar throughout the oestrous cycle with the highest concentration occurring on the afternoon of the pro-oestrous. Brown-Grant et al. (1970) had earlier shown that the oestradiol surge precedes the ovulatory surge of LH/FSH. The pre-ovulatory increase of progesterone is coincident with the surge of LH/FSH on the afternoon of pro-oestrus (Barraclough et al., 1971).

The aim of this preliminary study was to see whether we could obtain profiles for plasma steroid levels comparable with those of previous authors using our radioimmunoassay systems.

Sixteen female adult Wistar rats weighing over 200 g were placed in four cages in a lighting regime of 14 h light per day (illuminated between 08.00 h and 22.00 h). Vaginal smears were taken daily and only animals showing at least three consecutive 4-day cycles were used. Animals were killed by injecting sodium pentobarbitone (60 mg/kg body weight) between 15.30 and 16.30 h. Peripheral blood was collected by cardiac puncture into lightly heparinized syringes, centrifuged and the plasma stored at -20^{o}C until assayed. Samples were extracted with ether and the dried extract assayed for oestradiol, testosterone and progesterone using the radioimmunoassays already described. The result of this study is shown in Table 3.4.

Beginning on the afternoon of di-oestrus-1, there was a sustained rise in the plasma levels of oestradiol. The maximum values were obtained on the afternoon of pro-oestrus; these values being significantly different from other times studied. Testosterone demonstrated a similar pattern of secretion to oestradiol throughout the oestrous cycle with the highest concentrations occurring at 16.00 h of pro-oestrus. These high levels returned to basal values on the afternoon of oestrus. Like oestradiol and testosterone, the maximum concentrations of progesterone were obtained from plasma samples collected on the afternoon of pro-oestrus, while the lowest progesterone concentrations of the cycle were obtained on the afternoon of di-oestrus-2.

Table 3.4 Peripheral plasma concentration of oestradiol, testosterone and progesterone during the 4-day oestrus cycle of the wistar rat.

Stage of Cycle	Time	No. of Rats (n)	[1] Oestradiol Concentration ($\times 10^{-14}$ moles/ml)	[1] Testosterone Concentration ($\times 10^{-13}$ moles/ml)	[1] Progesterone Concentration ($\times 10^{-13}$ moles/ml)
Dioes-trus-1	16.00	4	3.85 ± 0.45^{c}	2.57 ± 0.24^{b}	18.25 ± 1.29^{a}
Dioes-trus-2	16.00	4	10.66 ± 0.90^{b}	3.59 ± 0.94^{b}	12.95 ± 0.49^{b}
Pro-oestrus	16.00	4	25.05 ± 4.92^{a}	4.10 ± 1.16^{a}	22.15 ± 0.36^{a}
Oestrus	16.00	4	2.18 ± 0.23^{c}	2.36 ± 0.18^{b}	15.90 ± 2.68^{b}

The experiment was performed on animals which had shown at least three consecutive 4-day cycles. Animals were sacrificed at times indicated in the table. Peripheral blood collected by cardiac puncture under sodium pentobarbitone anaesthesia was centrifuged and the plasma extracted with ether. The dried extract were assayed for oestradiol, testosterone and progesterone using our radioimmunoassay as described, in section 3.4.

[1] The data was examined by analysis of variance and the means compared by Duncan's multiple range test.

abc Mean values on any vertical column without a common superscript are significantly different ($p < 0.05$)

The present results are consistent with the oestradiol profile previously reported for peripheral plasma (Brown-Grant et al., 1970; Dupon and Kim, 1973) during the rat oestrous cycle, except that the latter authors observed highest oestradiol levels on the morning of pro-oestrus. Our oestradiol levels at pro-oestrus (25.0 $\pm$ 4.9 x 10^{-14} moles/ml) were compatible in absolute terms with 52.4 $\pm$ 6.0 pg/ml (19.0 $\pm$ 2.2 x 10^{-14} moles/ml) reported by Dupon and Kim (1973). Also testosterone levels at pro-oestrus (4.1 $\pm$ 1.0 x 10^{-13} moles/ml) compare well with 179.0 $\pm$ 36.1 pg/ml (6.2 $\pm$ 0.16 x 10^{-13} moles/ml) reported by the same authors. However, our progesterone levels at pro-oestrus (22.1 $\pm$ 0.36 x 10^{-13} moles/ml) were less than the 3.2 ng/ml observed by Barraclough et al., (1971) at 14.00 h of the day of pro-oestrus. (No samples were taken by these authors at 16.00 h). Since we have no data on the time interval between 16.00 h of pro-oestrus and 16.00 h of oestrus, we do not know whether higher levels of progesterone might have been observed at 24.00 h of oestrus, as reported by Barraclough et al., (1971). A more regular sampling may be needed if one wants to establish the precise time of maximum secretion of the three steroids. However, the present study was aimed at establishing the point of similar absolute values in peripheral plasma levels of oestradiol, testosterone and progesterone during the rat oestrous cycle, and this has been shown to be the case.

3.8 THE RELATIONSHIP BETWEEN STEROIDS ACCUMULATING IN THE MEDIUM AND THE LEVELS OF STEROIDS IN THE OVARIES OF PMSG PRIMED IMMATURE RATS: EFFECT OF HCG AND CYCLOHEXIMIDE

It has been reported that the ratio of the amount of steroids accumulating in the medium and the levels of steroids in the rats' Graafian follicles varied for different steroids (Lieberman, Barnea, Bauminger, Tsafriri, Collins and Lindner, 1973). It has also been shown that there is a consistent relationship between the rate of accumulation of steroids in the follicles and the release of the steroids into the medium (Lindner et al., 1974). The aim of this preliminary study was therefore to examine whether sampling the medium could be used to monitor the steroidogenic responses of the ovary to gonadotrophin (HCG) and the effect of an inhibitor of protein synthesis (cycloheximide).

Method

28-day old PMSG-primed rats were killed 48 h after PMSG had been injected as previously described. The ovaries were removed and placed individually in culture dishes containing varying concentrations of HCG (0 - 10.0 i.u./ml) or HCG (1.0 i.u./ml) with cycloheximide (25 µg/ml) and incubated for 4 h in KRB supplemented with glucose and glutamine as described earlier.

At the end of 4 h incubation period, the ovaries were quickly removed into 25 ml homogenizing tubes containing 1 ml acetone to stop all enzymatic reaction. Aliquots of the incubation medium were withdrawn, extracted with ether and the dried extract stored at -20^{o}C for subsequent determination of

steroid content. The ovaries were homogenized in 2 ml Kreb's bicarbonate buffer at pH 7.4, before extraction with 6 ml ether. The dried ethereal extracts were then assayed for oestradiol, testosterone and progesterone using the specific radioimmunoassays previously described.

Results and Discussion

The relationship between steroid concentration in the medium and tissue is shown in Table 3.5. A highly significant correlation between these two parameters was observed ($r > 0.96$, $P < 0.01$) for each of the steroids studied. However, as can be seen from Table 3.5 the extent of accumulation of progesterone in the tissue relative to the medium is substantially greater than that observed for oestradiol or testosterone. This is also reflected in the slopes from the linear regression equations relating tissue levels of oestradiol, testosterone and progesterone to their corresponding levels in the medium.

Since the accumulation of oestradiol, testosterone and progesterone in the medium appear to reflect changes in the concentration of these steroids in the ovarian tissue occurring in response to HCG in the presence or absence of cycloheximide, assays on small samples of the medium were routinely used in subsequent studies of steroidogenic response of the ovary to gonadotrophins and inhibitors.

Legend to Table 3.5

The experiment was performed with ovaries harvested from 28 day old rats previously primed with PMSG (5 iu) for 48 h. The ovaries were incubated for 4 h in unsupplemented medium (control) and in medium containing HCG (1.0 - 10.0 iu/ml) or in medium containing HCG (1.0 iu/ml) plus cycloheximide (25 µg/ml). After the incubation, aliquots of the medium were withdrawn and extracted with ether (as described in the text). Similarly ovarian homogenates were subjected to ethereal extraction. The steroids extracted from the medium, or from the tissues were assayed for oestradiol, testosterone and progesterone using our specific radioimmunoassays already described. Results are expressed as means ± SEM of four incubations.

Constants for the linear regression equations relating tissue and medium concentrations of steroids were calculated by the method of least squares using a Hewlett-Packard digital calculator.

Table 3.5 Relationship between steroids accumulating in the medium and the levels of steroids in the ovaries of PMSG primed immature rats: the effect of HCG in the presence or absence of cycloheximide.

Treatment	Oestradiol (pmole)			Testosterone (pmole)			Progesterone (pmole)		
	Medium M	Tissue T	Ratio M/T	Medium M	Tissue T	Ratio M/T	Medium M	Tissue T	Ratio M/T
PMSG-primed ovary (control)	1.58 ± 0.23	2.08 ± 0.26	0.76 ± 0.08	5.0 ± 0.5	6.10 ± 0.55	0.82 ± 0.09	1.53 ± 0.16	4.08 ± 0.46	0.38 ± 0.04
Primed ovary + 1.0 iu HCG	2.08 ± 0.12	2.83 ± 0.31	0.73 ± 0.12	7.63 ± 0.58	9.3 ± 0.8	0.78 ± 0.05	2.08 ± 0.15	5.35 ± 0.45	0.39 ± 0.04
Primed ovary + 2.5 iu HCG	2.41 ± 0.31	2.9 ± 0.3	0.83 ± 0.11	9.20 ± 0.97	11.4 ± 1.1	0.82 ± 0.10	2.30 ± 0.25	6.20 ± 0.51	0.37 ± 0.04
Primed ovary + 5.0 iu HCG	2.80 ± 0.41	3.4 ± 0.4	0.82 ± 0.08	10.25 ± 1.50	12.53 ± 1.41	0.82 ± 0.08	2.80 ± 0.38	7.8 ± 0.7	0.36 ± 0.05
Primed ovary + 7.5 iu HCG	3.25 ± 0.25	4.0 ± 0.1	0.81 ± 0.11	10.8 ± 0.9	13.33 ± 1.20	0.81 ± 0.07	3.28 ± 0.13	9.18 ± 1.30	0.36 ± 0.04
Primed ovary + 10 iu HCG	3.7 ± 0.4	4.05 ± 0.50	0.81 ± 0.09	11.5 ± 1.2	14.6 ± 1.1	0.81 ± 0.08	3.63 ± 0.45	11.0 ± 0.9	0.33 ± 0.04
Primed ovary + 1.0 iu HCG + 25 µg/ml cycloheximide	1.39 ± 0.19	2.53 ± 0.28	0.75 ± 0.11	7.18 ± 0.55	9.0 ± 0.6	0.79 ± 0.11	1.90 ± 0.20	4.75 ± 0.48	0.40 ± 0.05
r (correlation coefficient)		0.96			0.99			0.99	
slope		1.0			1.2			3.3	

CHAPTER 4

COMPARISON OF THE BIOLOGICAL ACTIVITY OF FOUR GONADOTROPHIN PREPARATIONS, USING THE STEROIDS SECRETED BY LEYDIG CELLS OR GRANULOSA CELLS AS AN INDEX OF THEIR POTENCIES

4.1 INTRODUCTION

It has become common practice to substitute human choronic gonadotrophin (HCG) for luteinizing hormone (LH) in experiments, and to use pregnant mares' serum gonadotrophin (PMSG) in place of follicle stimulating hormone (FSH). We have used PMSG to 'prime' the ovaries of immature rats (Ebong and Peddie, 1979), and HCG to stimulate Leydig cells or ovarian cells *in vitro* (Brain, Peddie and Taylor, 1980; Cole and Peddie, 1980; Richardson and Peddie, 1980).

There are some references in the literature on the comparative LH- and FSH-like activities of PMSG, based on receptor binding studies with these hormones (Stewart, Allen and Moor, 1976; Newcomb, Christie, Rawson, Walters and Bonsfield, 1979), but we are more concerned with comparisons of their ability to cause steroid secretion.

It has been demonstrated that Leydig cells isolated from the adult rat testes secrete predominantly testosterone and that they bind HCG and LH but not FSH *in vitro* (Dufau and Catt, 1975). It has also been established that FSH but not LH causes oestradiol secretion from granulosa cells isolated from the preovulatory follicles of PMSG primed immature rats (Fortune and Armstrong, 1978).

We have used methods for preparing and incubating cells from testes and ovaries similar to those used by these authors,

to compare the biological activity of ovine FSH, ovine LH, PMSG and HCG.

4.2 METHODS

Immature female rats, and adult male rats (250 g) were used from the same colony as described previously (section 2.1). They were killed by cervical dislocation and gonads removed for subsequent collection of cells.

Preparation of granulosa cells

The method used was that described previously (section 2.11), based on the method of Hillier, Van der Boogard, Reichert and Van Hall (1980). The cells incubated for 4 h in Kreb's ringer bicarbonate buffer (KRB) pH 7.4, supplemented with glucose and glutamine, gassed with 95% O_2:5% CO_2. 1 x 10^{-7}M testosterone was present in each incubation tube as substrate for the aromatization activity. The gonadotrophins were added to the incubation at time zero, and cells were used at a concentration of 1 x 10^5 cells/ml medium. The incubates were centrifuged at the end of the experiment and the supernatant assayed for oestradiol, by the specific radioimmunoassay described previously (section 3.4).

Preparation of Leydig cells

Leydig cells were separated from other testicular cells as described previously (section 2.12) based on the method of Janszen, Cooke, Van Driel and Van der Molen (1976). They were suspended, after washing in KRB supplemented with glucose and glutamine, at a concentration of 0.4 x 10^5 cells/ml, and

incubated for 4 h in the presence or absence of the gonadotrophins. No exogenous steroids were added to the incubates. The secreted testosterone was measured by the radioimmunoassay already decribed in section 3.4.

Gonadotrophin preparations

The four gonadotrophic hormone preparations used were:

1. Ovine FSH kindly supplied by NIAMDD, through Dr. A.R. Parlow. Batch No. NIAMDD-oFSH ≠ 13, (with a reported biopotency of 15 u/mg. Contamination by LH = 0.05 times NIH-LH-SI).
2. Ovine LH, also donated by NIAMDD. Batch No. NIAMDD-LH ≠ 21; (with a reported biopotency of 2.5 u/mg. Contamination by FSH less than 0.5% by weight).
3. 'Pregnyl', HCG, batch No. 6299 (containing 3250 iu/mg protein) was supplied by Organon Ltd.
4. PMSG, batch No. 87C - 04831 (containing 1970 iu/mg protein) was obtained from Sigma Corporation Ltd.

4.3 RESULTS

Oestradiol secretion from granulosa cells

FSH caused a marked increase in the oestradiol secreted, from 4.95 ± 0.25 pmoles/10^5 cells/4 h with no added FSH, to 7.48 ± 0.42 pmoles/10^5 cells/4 h with the addition of 10 µg FSH/ml medium. The half-maximal value of FSH (ED_{50}) was approximately 0.1 µg/ml FSH, and maximal stimulation was achieved with 1.0 µg/ml. In contrast, neither LH nor HCG stimulated oestradiol secretion over the dose ranges 0.1 µg/ml to 10 µg/ml LH or 0.10 iu/ml to 100 iu/ml HCG (equivalent to

0.05 - 5 µg protein/ml). However, PMSG caused a linear increase in oestradiol secretion, over the range 0.1 iu/ml to 100 iu/ml (equivalent to 0.307 - 307 µg protein/ml), with an ED_{50} of approximately 10 iu/ml or 30.7 µg protein/ml. (Figure 4.1).

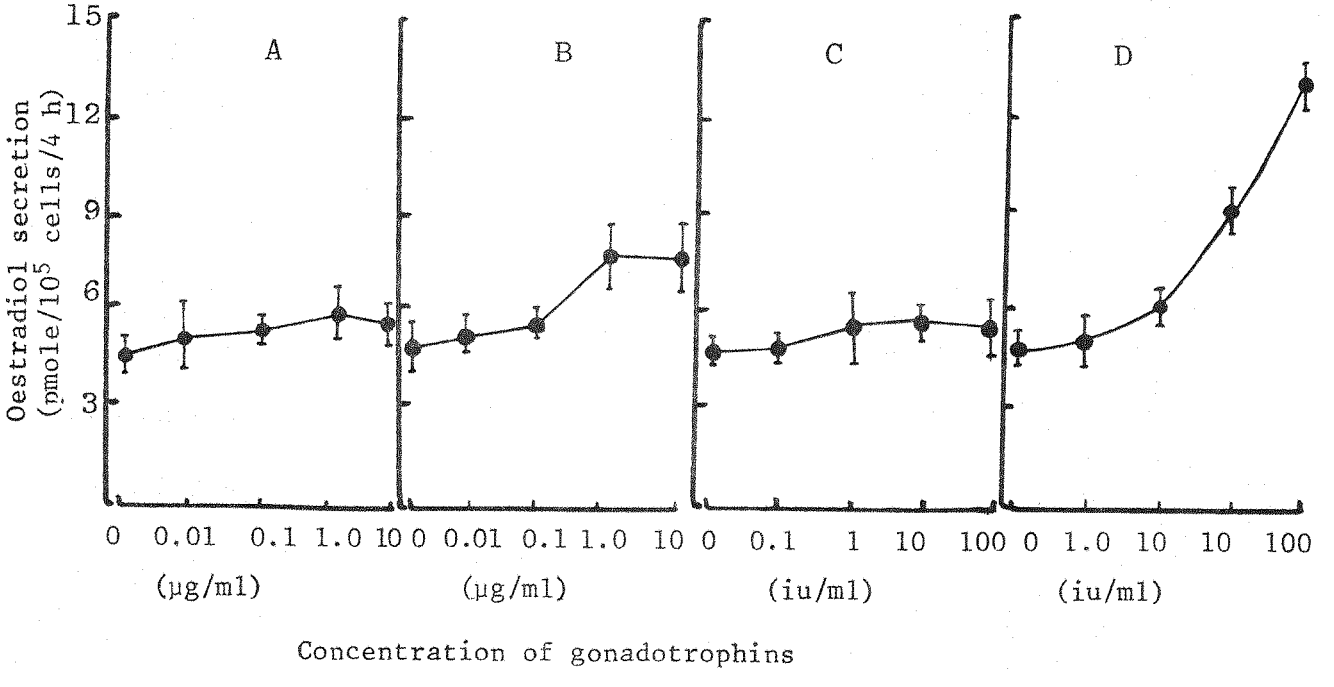
Testosterone secretion from Leydig cells

LH caused a linear increase in testosterone secretion from the isolated Leydig cells at levels between 0 and 1.0 µg/ml, with a maximal stimulation at 1.0 µg/ml, and an ED_{50} of approximately 0.1 µg/ml. HCG also caused a marked increase in the level of testosterone secreted, from 2.02 $\pm$ 0.18 pmoles/10^5 cells/4 h in the absence of gonadotrophin, to 7.2 $\pm$ 0.4 pmoles/10^5 cells/4 h, with the addition of 10 iu/ml (equivalent to 5 µg/ml protein).

In contrast, FSH over a dose range 0 to 10 µg/ml medium, caused an 80% increase in steroid secretion from 2.10 $\pm$ 0.32 pmoles/10^5 cells/4 h in absence of exogenous gonadotrophin, to 3.96 $\pm$ 0.64 pmoles/10^5 cells/4 h at 1.0 µg/ml FSH, with no subsequent significant rise at higher levels of added gonadotrophin. Similarly, PMSG caused only a 50% increase in testosterone secretion, with maximal levels achieved with 100 iu PMSG/ml (Figure 4.2).

Figure 4.1

Oestradiol secreted by granulosa cells isolated from the pre-ovulatory follicles of immature rats injected with 5.0 i.u. pregnant mares' serum gonadotrophin 48 h before sacrifice. The granulosa cells (1×10^5 cells/tube) were incubated in Kreb's Ringer Bicarbonate buffer, pH 7.4, for 4 h at 37°C, gassed with 95% O_2 5% CO_2, with the addition of either (A) ovine luteinizing hormone (B) ovine follicle stimulating hormone (C) human chorionic gonadotrophin or (D) pregnant mares' serum gonadotrophin, at the dose levels indicated on the abscissae.
Results are shown as means ± SEM of four incubations.



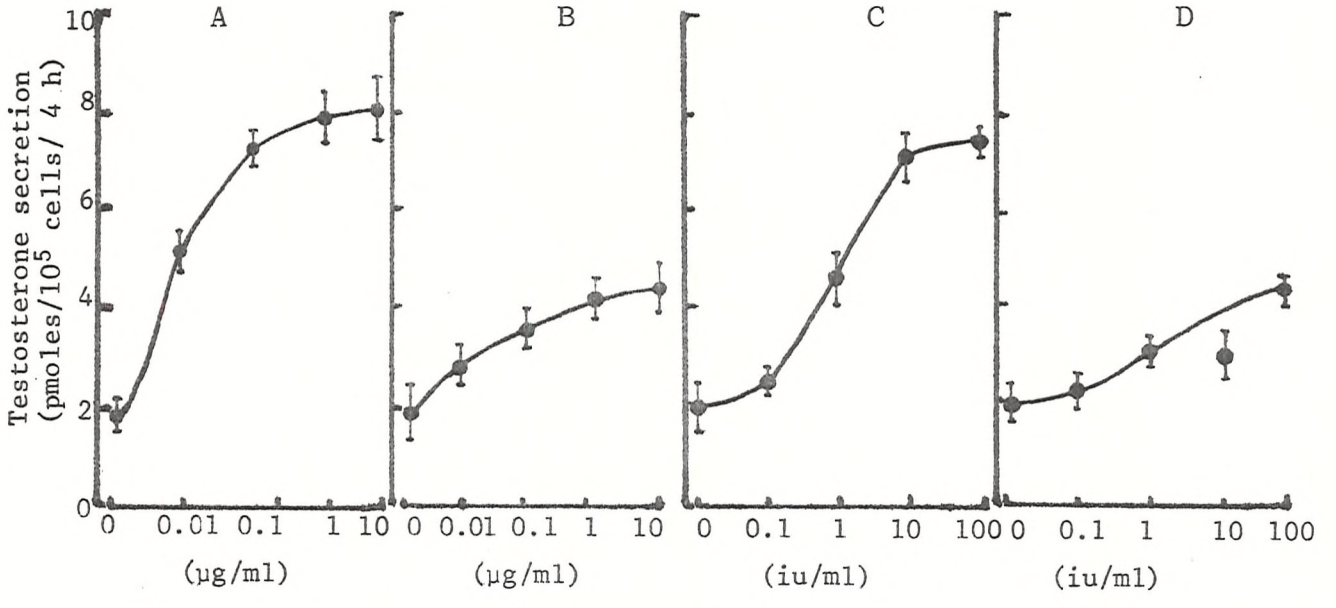
In this experiment t_o was not measured.

Figure 4.2

Testosterone secreted by Leydig cells isolated from adult rat testes in response to (A) ovine luteinizing hormone, (B) ovine follicle stimulating hormone, (C) human chorionic gonadotrophin or (D) pregnant mares' serum gonadotrophin.

The Leydig cells were incubated for 4 h at 37°C in Kreb's Ringer Bicarbonate buffer, pH 7.4, gassed with 95% O_2 5% CO_2 at a concentration of 0.4×10^5 cells/ml incubation medium.

Results are shown as means ± SEM of four incubations



4.4 DISCUSSION

These results indicate, in common with previously reported results, that FSH causes a stimulation of oestradiol secretion by granulosa cells isolated from PMSG-primed immature rats' ovaries, in the presence of exogenous testosterone. The response was dose-related. However, this effect was observed within 4 h in the present experiments, while after 24 h culture of granulosa cells isolated from hypophysectomized immature rat in the presence of 0.5 μM testosterone, Dorrington, Moon and Armstrong (1975) did not observe any marked stimulation of oestradiol secretion by FSH. A likely explanation for this difference is that prior exposure of the PMSG-primed immature rats to gonadotrophins *in vivo* resulted in stimulation of aromatase activity in granulosa cells (Suzuki, Kawakura and Tamaoki, 1978) which persisted during the incubation period.

The stimulation of oestradiol secretion reached a much higher level in the presence of PMSG, and with the largest amount used (100 iu/ml) had not reached a maximal response. This could be due to the inherent LH or HCG like activity of the hormone, which might stimulate and increase formation of progesterone and possibly testosterone (in thecal cells which have been a contaminant in the preparation) and hence increase the supply of substrate for aromatization. In addition PMSG has a longer half life in the circulation than FSH, and may be more active over a longer period than FSH, even *in vitro*.

Neither LH nor HCG caused any marked stimulation of oestradiol secretion from the isolated granulosa cells, an observation which is consistent with previously published reports (Dorrington et al., 1975; Fortune and Armstrong, 1978). However, these results differ from the observations of Armstrong,

Goff and Dorrington (1979) who reported that granulosa cells isolated from immature rats primed with 12 iu PMSG 48 h previously responded to both FSH and LH. Whether this difference is attributable to the high dose of PMSG used by Armstrong et al. (1979) to prime the animals, or to other factors such as differences in culture conditions or in the LH/HCG receptors which have been identified on granulosa cells (Channing and Kammerman, 1974; Zeleznik, Midgley and Reichert, 1974), cannot be deduced from the present studies.

Both LH and HCG caused an increased secretion of testosterone from Leydig cells. This is compatible with previously reported studies (Dufau and Catt, 1975; Janszen et al., 1976). In contrast, FSH and PMSG caused only modest increases in the amount of testosterone secreted. This is not surprising since Leydig cells are poor in FSH receptors while LH/HCG receptor sites are abundant on the testicular Leydig cells (Dufau and Catt, 1975).

The ability of LH and HCG to stimulate significant testosterone secretion from the Leydig cells isolated from rat testes and the ability of FSH and PMSG to stimulate oestradiol secretion from the granulosa cells of PMSG-primed immature rat ovaries provides a useful *in vitro* bioassay for comparing the biological activities of these gonadotrophins. "As a bioassay, the steroidogenic response of Leydig cells and granulosa cells provides a highly sensitive method, which is rarely attainable by conventional bioassays" (Dufau and Catt, 1975). Previous bioassays for LH and HCG have depended upon the responses of secondary target tissues to steroids produced by the gonad *in vivo* [(e.g. ventral prostate of the hypophysectomized male rats (McArthur, 1952) and the uterus of immature female rat (Albert,

1956)] or upon depletion of ascorbic acid in the ovary of immature pseudo-pregnant rats (Parlow, 1961). Such assays are laborious and extravagant of animals while the present studies provide a highly specific and sensitive method with less cost.

CHAPTER 5

STEROIDS SECRETED BY OVARIES OF IMMATURE RATS PRETREATED WITH PMSG OR SALINE: EFFECT OF HCG IN VITRO

5.1 INTRODUCTION

The pattern of steroids secreted by individual Graafian follicles of rats, which have been harvested before the endogenous LH-FSH surge on the day of pro-oestrus, is well documented (Lindner, Tsafriri, Lieberman, Zor, Koch and Bauminger, 1974; Hillensjo, Bauminger and Ahren, 1976; Hamberger, Hillensjo and Ahren, 1978). These workers found that the addition of LH *in vitro* resulted in an initial stimulation of steroid production, followed after 4 - 6 h by a decrease in androgen and oestrogen formation. In contrast, progesterone accumulation continued and subsequently increased. A similar transition in the pattern of follicular steroidogenesis seems to occur *in vivo* as a result of the endogenous LH secretion (Katz and Armstrong, 1976; Hillensjo, Hamberger and Ahren, 1977). Thus the aim of the present study was to establish the profile of oestradiol, testosterone and progesterone both in the plasma and secreted by the ovaries of immature rats pretreated with PMSG or saline. Immature female rats in which follicular development had been induced by PMSG (5 iu), were used in this study.

Follicular development initiated by PMSG is similar to the development of follicles during the pro-oestrus phase of the cycle of the adult rat (Hillensjo, Bauminger and Ahren, 1976). PMSG enhances ovarian enzyme activities relevant to oestrogen production (Suzuki, Kawakura and Tamaoki, 1978),

resulting in an increased secretion of oestradiol-17β (Sashida and Johnson, 1976) and thereafter, the oestradiol secreted causes the LH-FSH surge (Costoff, Eldridge and Mahesh, 1974). The effects of the endogenous LH-FSH surge on the pattern of steroids secreted by the ovary following morphological changes induced by PMSG were examined.

5.2 METHODS

In a preliminary study, 3 doses of PMSG (1.0, 3.0 or 5.0 iu/ml) in 0.1 ml saline, or 0.1 ml saline, (controls) were injected subcutaneously (s.c.) at 10.00 h into 25-day old rats. 48 h after the PMSG injection, the rats were killed by cervical dislocation. The ovaries and uteri were removed, cleared of surrounding connective tissue and weighed. 5 iu PMSG was the minimum effective dose which was found to cause a significant increase in both uterine and ovarian weights (see Results section, Table 5.1).

In the second experiment, 5 iu PMSG in 0.1 ml saline, or 0.1 ml saline, were injected sc at 10.00 h into 25-day old animals. Groups of 4 of these PMSG-primed animals and the corresponding saline-injected control rats were killed at 10.00 h on days 26 or 27, or at 02.00, 06.00 and 10.00 h on day 28. At the time that the animals were killed, blood was collected by cardiac puncture into a lightly heparinized syringe, centrifuged and the plasma was stored at -20°C until it was assayed. In addition the ovaries and uteri were removed, cleared of the surrounding tissues, visually examined for the development of large follicles and then weighed. The oviducts were examined under the dissecting microscope for the

presence of ova, as an indication of whether ovulation had occurred.

The ovaries were incubated in KRB (supplemented with glucose and glutamine as described previously) with and without added HCG (1.0 iu/ml). This dose of HCG was selected after a preliminary experiment had shown that this was the minimum dose which caused a significant stimulation of oestradiol, testosterone and progesterone secretion by these incubated ovaries. The steroids accumulating in the medium were measured at times 0, 1, 2, 3 and 4 h after adding the HCG, by removing 200 µl in duplicate for each of the steroids to be assayed. The equivalent volume of incubation medium with or without HCG was replaced and the subsequent dilution corrected for. The samples were stored at -20°C until assayed for oestradiol, testosterone and progresterone using the specific radioimmunoassays described in Chapter 3.

The third experiment was designed to investigate whether an alteration in the profile of steroid secretion occurred after the endogenous LH-FSH surge. The effect of sodium pentobarbitone on the profile of steroid secretion was also examined. The experimental protocol was similar to that previously described by Hillensjo et al. (1976). 4 groups of 4 animals were injected with 5 iu PMSG on day 25 and animals killed at different times on day 27 or day 28. The first group of rats in this experiment, were killed between 09.00 and 10.00 h i.e., before the expected endogenous LH surge (Hillensjo et al., 1976). The second group were killed between 20.00 and 21.00 h, after the expected endogenous LH surge. The third and fourth groups were injected i.p. with sodium pentobarbitone (35 mg/kg body weight) at 14.00 h. The third group were killed between 20.00 and

21.00 h on day 27, while the fourth group were killed between 09.00 and 10.00 h on day 28. The ovaries and uteri were removed and treated as previously described for experiment 2.

In the fourth experiment, 7 groups of 2 animals were killed by cervical dislocation at times 0, 24, 48 and 72 h after the PMSG (5 iu) or 0.1 ml saline (control) injection on day 25. The ovaries were removed, cleared of all connective tissue and fixed in Bouin's solution for the subsequent histological processing as described in Chapter 2.

5.3 RESULTS

Experiment 1

In Table 5.1 are shown the changes in ovarian and uterine weights recorded after treating 25-day old female rats with the 3 different doses of PMSG (1.0, 3.0 and 5.0 iu/ml). 1.0 iu of PMSG caused a small but not significant increase in ovarian and uterine weights when compared with saline treated controls. 3.0 iu PMSG caused a significant increase in uterine weight ($P < 0.02$) when compared to the saline treated controls, but there was no significant increase in ovarian weight with this dose of PMSG. In contrast, 5.0 iu PMSG caused a significant increase in both uterine and ovarian weights ($p < 0.01$) when compared with the saline treated controls. 5 iu PMSG was therefore used in subsequent studies since it was judged to be the minimum effective dose to cause the growth of uteri and ovaries.

Experiment 2a

Ovarian weights did not increase significantly above those recorded on day 25 until 48 h after PMSG had been injected (i.e. until 10.00 h on day 27). The increase in ovarian weight continued to day 28. An increase in the size of the follicles was already evident on gross inspection of the ovary on the morning of day 26, and by 10.00 h on day 27, the ovarian follicles were substantially enlarged with clusters of ripe follicles lying on the surface of the organ. By the morning of day 28, newly formed corpora lutea were seen.

The uterine weights increased earlier than the weights of the ovary, showing a significant increase above the controls by 10.00 h on day 26 ($P < 0.05$). A maximum increase was observed on day 27, followed by a dramatic decrease 24 h later ($P < 0.02$). These results are compared in Table 5.2.1.

All the animals examined on day 28 except those killed at 02.00 h had 8 to 12 ova in their oviducts (Table 5.2.2). No ova were observed on days 26 and 27. Saline treated control rats had small pale ovaries, and no ova in their oviducts, including those animals killed on day 28.

Experiment 2b

The injections of PMSG (5 iu) caused significant increases in plasma oestradiol, testosterone and progesterone as shown in Figure 5.1. Plasma oestradiol levels rose steadily following the injection of PMSG and reached maximum levels on day 27 ($P < 0.01$ when compared with the levels in control plasma of day 25 animals). 24 h later (day 28) oestradiol levels in the experimental animals were significantly lower ($P < 0.05$) than the levels found on day 25 or than those in saline treated

controls of day 25. Plasma oestradiol levels in the control rats did not change significantly over the same 72 h period, being at or below the limit of detection of the assay.

Changes in the plasma concentrations of testosterone after the PMSG or saline treatment are depicted in Figure 5.1.b. Testosterone levels rose within 24 h after PMSG had been injected on day 25. On day 27 significant increase had occurred in testosterone levels ($P < 0.01$)when compared with control plasma concentrations on day 25. The plasma testosterone levels decreased significantly on day 28, but had not fallen below the levels measured in the controls on day 25. The concentration of progesterone in the plasma (Figure 5.1.c) rose slightly 24 h after PMSG had been injected, then remained almost constant over the next 24 h (day 27). A significant increase ($P < 0.01$) in the plasma concentrations of progesterone was noted on day 28 compared with any other period examined.

Experiment 2c

The amounts of oestradiol, testosterone and progesterone accumulating in the medium containing the ovaries of immature rats primed with PMSG, or by the ovaries of comparable saline treated animals, in the presence or absence of HCG, are summarized in Table 5.3 Basal amounts of the three steroids ($3.8 \pm 0.5 \times 10^{-14}$ moles; $4.7 \pm 0.8 \times 10^{-13}$ moles; or $3.0 \pm 0.6 \times 10^{-12}$ moles/ml for oestradiol, testosterone and progesterone respectively) appeared to be secreted by the ovaries of 25-day old rats (not injected with PMSG). There was however, no stimulation when HCG (1.0 iu/ml) was added to these incubates, neither were the levels of steroid which acc-

umulated in the medium containing the ovaries from rats treated with PMSG 24 h previously, different from those in the media containing the ovaries of saline treated controls. However, 48 h after PMSG had been injected, a significant increase in the amounts of oestradiol and testosterone ($P < 0.01$) accumulating were observed both in the medium to which HCG had been added and in hormone-free medium, compared with saline treated controls. These steroids reached maximal levels at the end of the 4 h incubation. In contrast when ovaries of rats which had been treated with PMSG 72 h previously were incubated in the presence of HCG, the amount of oestradiol which accumulated in the medium declined sharply ($2.8 \pm 0.5 \times 10^{-14}$ moles/ ml) to levels lower than those observed when the ovaries of saline treated control rats ($5.1 \pm 0.8 \times 10^{-14}$ moles/ml) were incubated under the same conditions. Similarly, testosterone levels in the incubation medium which contained the ovaries from rats which had been treated with PMSG 72 h previously, and to which HCG had been added, showed a significant reduction ($P < 0.02$) compared with the testosterone levels attained in the medium which contained the ovaries from rats injected with PMSG 48 h previously and to which HCG had been added. These differences were maximal at the end of the 4h incubation period. However, in contrast to oestradiol, testosterone levels in the medium at the end of the 4 h incubation period were still higher than the levels attained by the saline treated controls ($P < 0.05$).

The levels of progesterone in the incubation medium containing the ovaries from rats which had been treated with PMSG 48 h previously, in the absence of exogenous HCG, were exceedingly low ($4.0 \pm 0.7 \times 10^{-12}$ moles/ml), values which were not significantly different from those attained in the

media containing the ovaries of saline treated rats. Similarly, the levels of progesterone which accumulated in the media containing the ovaries of rats treated with PMSG 72 h previously, in the absence of exogenous HCG, were also low ($12.9 \pm 0.1 \times 10^{-12}$ moles/ml). The addition of HCG to the medium increased the levels of progesterone significantly within 1 h ($20.1 \pm 2.0 \times 10^{-12}$ moles/ml) ($P < 0.01$) and by 4 h the increased levels of progesterone observed were 4 fold greater than those measured when ovaries from rats treated with PMSG 48 h previously had been similarly incubated.

In contrast there was no significant increase in the levels of oestradiol, testosterone or progesterone when HCG was added to the media containing ovaries of rats which had been injected with saline 72 h previously (Table 5.3).

Experiment 3

Figure 5.2 summarizes the amounts of oestradiol, testosterone and progesterone accumulating in the medium containing the ovaries of immature rats primed with PMSG or by the ovaries of comparable PMSG primed animals in which ovulation had been blocked by sodium pentobarbitone ('Sagatal'). A single injection of the barbiturate (35 mg/kg body weight) at 14.00 h on day 27 consistently blocked ovulation which was otherwise observed to have occurred on the morning of day 28 in the PMSG-primed animals (Table 5.2).

Incubates of ovaries harvested before the endogenous gonadotrophin surge, contained predominantly oestradiol and testosterone and only small amounts of progesterone. Adding HCG (1.0 iu/ml) to the medium increased the levels of all three steroids. The

oestradiol level in the medium in the presence of HCG was about 4 times higher than that in the control medium. The corresponding levels of testosterone and progesterone were 3 and 5 times above their control levels respectively.

Incubates of ovaries harvested after the endogenous LH-FSH surge contained small amounts of oestradiol and testosterone, without the addition of HCG, while the progesterone levels in the media were significantly greater than those observed before LH surge. Adding HCG to the incubation media which contained these ovaries had no effect on either oestradiol or testosterone levels, while the exogenous hormone stimulated a further increase in the progesterone levels. Figure 5.2 also illustrates that the steroids which accumulated in the media containing ovaries of the third experimental group in which the endogenous gonadotrophin surge had been blocked by sodium pentobarbitone, were similar to those of the first experimental group, both in the presence or absence of HCG. The ovaries from the fourth group of rats when incubated in the presence or absence of HCG secreted smaller amounts of the 3 steroids ($P < 0.01$) compared with the oestradiol and testosterone levels attained in the medium which contained the ovaries from rats injected with barbiturate (the third experimental group) and to which HCG had been added.

Experiment 4

Histological specimens from the ovaries of saline treated control animals and those treated with PMSG for 24, 48 and 72 h before sacrifice are contrasted in fig. 5.3. Ovaries from the

control animals were characterized by an abundance of small antral follicles with few layers of granulosa and thecal cells (Fig. 5.3.A). The ovaries of rats treated with PMSG 24 h previously contained medium sized antral follicles and a few large antral follicles, with distinctive layers of granulosa cells. Fully mature Graafian follicles were not observed at this stage (Fig. 5.3.B). 48 h after PMSG had been injected, the ovaries contained numerous pre-ovulatory Graafian follicles (Fig. 5.3.C). These follicles had thecal layers which were thicker than those observed in follicles from other treatment groups. The follicles also looked 'healthy' since neither the oocytes nor the well defined granulosa cells showed any signs of degeneration. In contrast, no 'healthy' Graafian follicles were found in ovaries of animals treated with PMSG 72 h previously. However, these ovaries did contain many corpora lutea which indicated that ovulation had occurred.

Table 5.1 Ovarian and uterine weight changes 48 hours after injecting 25-day old female rats with varying doses of PMSG.

	Dose of PMSG (iu)[b]			
	0	1	3	5
Ovarian Weight[a]	34.5 ± 1.8	36.1 ± 3.1	42.3 ± 6.6	50.5* ± 6.5
Uterine Weight[a]	68.0 ± 6.4	74.5 ± 5.8	114.7** ± 10.4	128.0** ± 6.9

[a] Organ weights are expressed as mg/100 g body weight and are presented as the mean ± SEM. n = 4/group

[b] Female rats were injected with various doses of PMSG s.c. or with the vehicle alone (0 Dose) at 10.00 h at 25 days of age. The rats were sacrificed at 10.00 h at 27 days of age.

* Indicates a significant increase in organ weights ($P < 0.02$) when compared with vehicle treated controls.

** Indicates significant increase in organ weights ($P < 0.01$) when compared with vehicle treated controls.

Table 5.2.1 Ovarian and uterine weights 24, 48 or 72 h after injecting 25-day old female rats with 5 iu PMSG or with saline.

Day of Autopsy		Intact rats treated with:	
		Saline	5 iu PMSG[b]
25	ovaries[a] (mg)	30.1 ± 1.7	30.5 ± 1.2
	uterus[a] (mg)	70.0 ± 3.2	70.7 ± 9.0
26	ovaries[a] (mg)	33.1 ± 2.1	38.0 ± 3.0
	uterus[a] (mg)	69.9 ± 4.1	117.5 ± 10.1*
27	ovaries[a] (mg)	33.3 ± 1.8	50.0 ± 3.5**
	uterus[a] (mg)	75.5 ± 6.5	130.0 ± 13.8**
28	ovaries[a] (mg)	35.4 ± 2.9	65.7 ± 2.8**
	uterus[a] (mg)	80.5 ± 5.8	110.1 ± 9.5*

Immature female rats were injected with PMSG (5 iu) or with saline (0.1 ml) on day 25 of life. Groups of 4 experimental rats and their comparable saline treated controls were killed at 10.00 h on the days indicated in the table. Uteri and ovaries were removed, cleared of connective tissue and weighed.

[a] Organ weights are expressed as mg/100g body weight and are presented as the mean ± SEM. (n = 4 rats/group)

* Indicates a significant increase in organ weights ($P < 0.05$) when compared to day 25 PMSG-injected animals.

** Indicates a significant increase in organ weights ($P < 0.01$) when compared to day 25 PMSG-injected animals.

Table 5.2.2 Time course of the appearance of ova* in the oviduct following an injection of PMSG (5 iu) on day 25 of life in immature rats.

Treatment	Autopsy		No of ova (range)
	Day	Time	
PMSG, day 25	26	10.00	
Saline, day 25	25	10.00	
PMSG, day 25	27	10.00	
Saline, day 25	27	10.00	
PMSG, day	28	02.00	
		06.00	8 - 11
		10.00	9 - 12
Saline, day 25	28	02.00	
		06.00	
		10.00	

Immature female rats were injected with PMSG (5 iu) or with saline (0.1 ml) on day 25 of life. Groups of 4 experimental rats and their comparable saline treated controls were killed at 10.00 h on the days indicated in the table. Uteri and ovaries were removed, cleared of connective tissue and weighed.

*Uteri were examined as described in the text and the presence of ova was recorded.

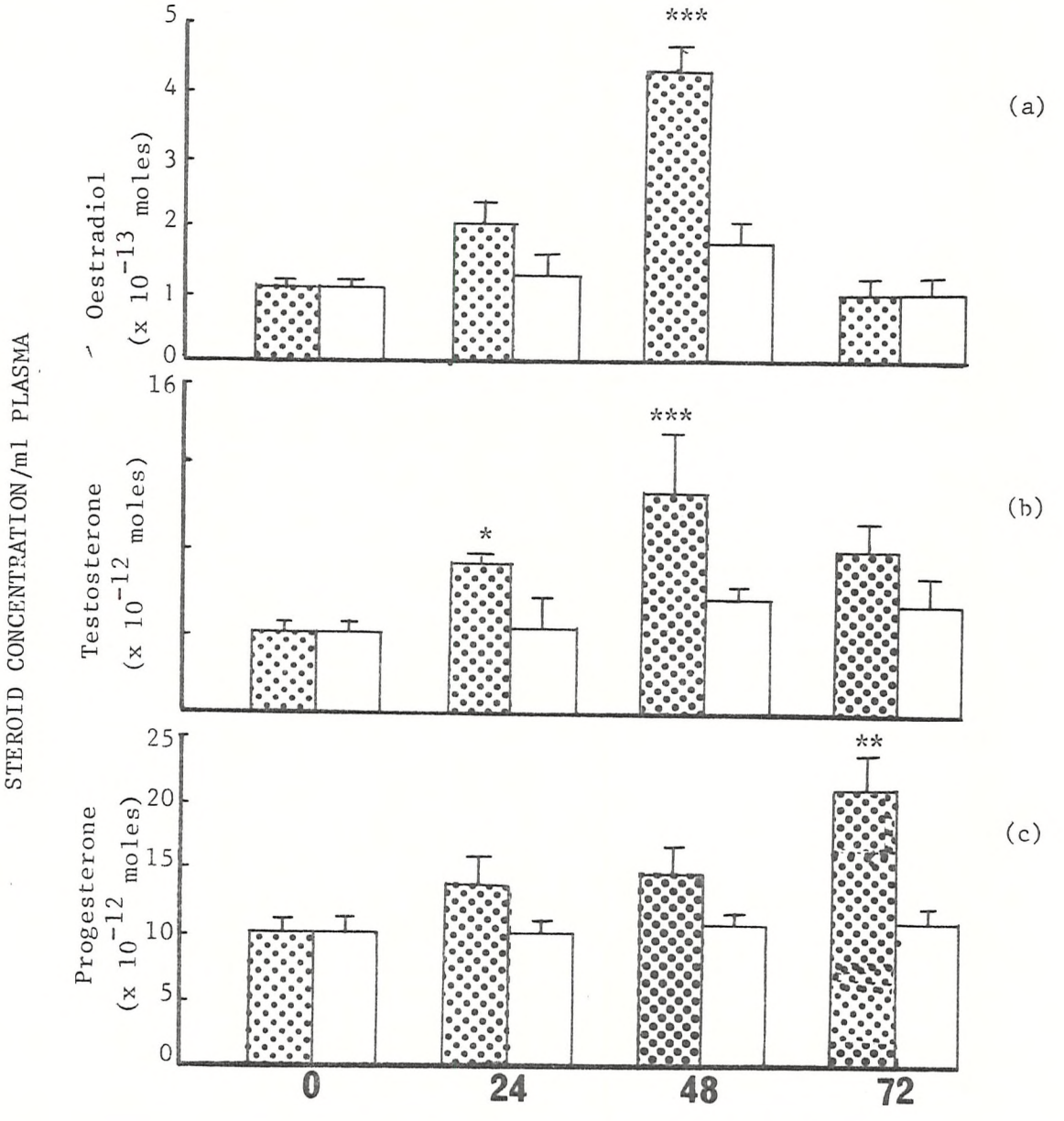
Legend Table 5.3

The experiment was performed with ovaries which had been removed from rats at the times indicated in the table after the animals had been injected with PMSG (5 iu/ml) or saline (0.1 ml; control) on day 25. The ovaries were incubated in hormone free medium or in medium containing 1.0 iu HCG. Oestradiol, testosterone and progesterone accumulating in the medium at various time intervals, (0 - 4 h) were measured by radio-immunoassay as previously described. Results are shown as means ± SEM of 4 incubations.

Table 5.3 Steroid secretion profile by the ovaries of immature rats pretreated with PMSG or saline (control) in the presence or absence of HCG, in vitro.

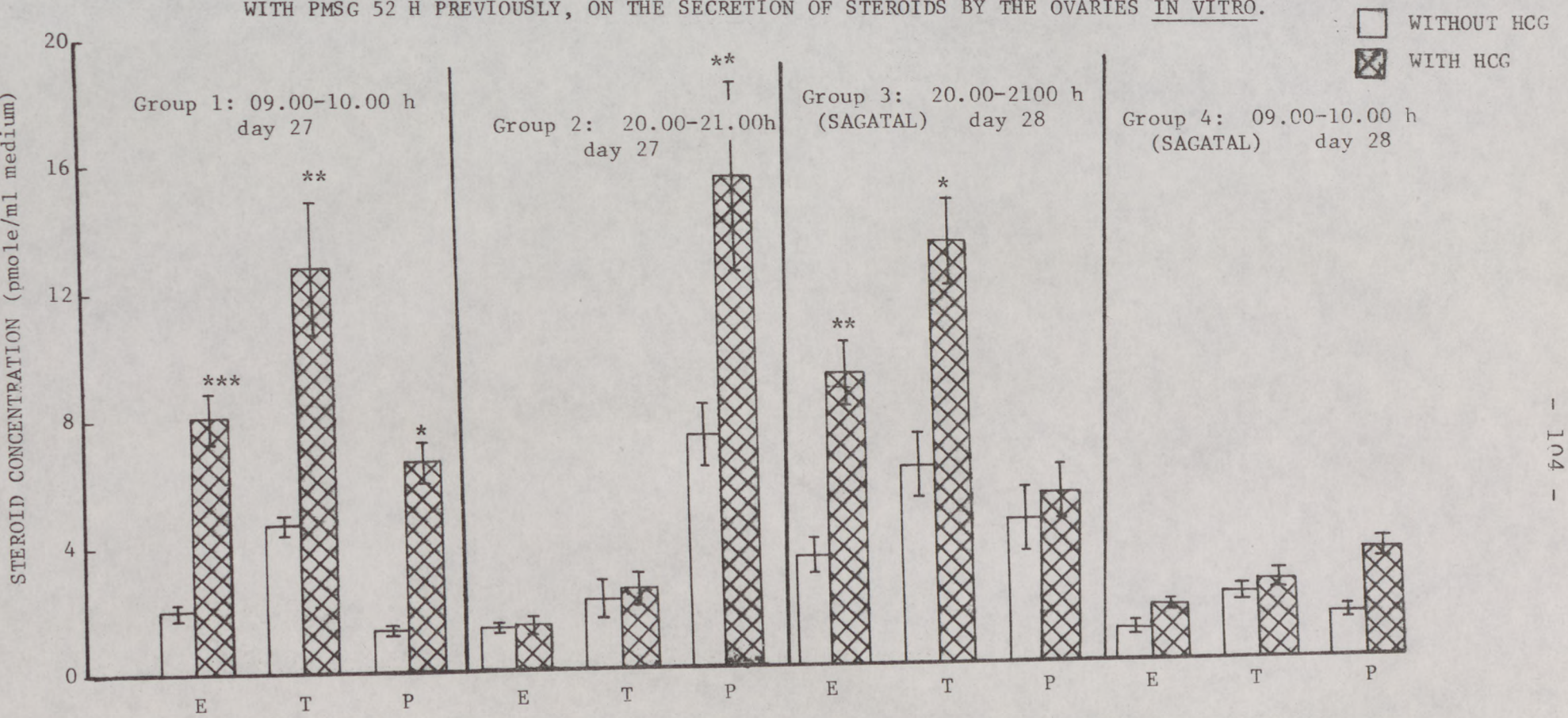
Age of Animal (days)	Time after PMSG or Saline Treatment (h)	Duration of Incubation (h)	STEROID CONCENTRATION (ml/medium) (Primed ovaries without HCG) Oestradiol (x 10^{-14} moles)	Testosterone (x 10^{-13} moles)	Progesterone (x 10^{-12} moles)	(Primed ovaries with HCG) Oestradiol (x 10^{-14} moles)	Testosterone (x 10^{-13} moles)	Progesterone (x 10^{-12} moles)
25	0	0	3.7 ± 0.7	4.6 ± 0.8	3.1 ± 0.6	3.8 ± 0.5	4.7 ± 0.8	3.0 ± 0.6
26	24	0	4.0 ± 0.5	4.9 ± 0.6	3.0 ± 0.5	4.1 ± 0.2	5.1 ± 0.7	3.1 ± 0.5
		1	4.2 ± 0.2	3.9 ± 0.4	4.0 ± 0.8	4.5 ± 0.5	7.6 ± 0.9	4.4 ± 0.8
		2	3.9 ± 0.5	5.8 ± 1.0	3.8 ± 0.7	4.9 ± 0.7	7.9 ± 1.0	4.5 ± 0.7
		3	3.9 ± 0.5	5.9 ± 0.7	4.1 ± 0.4	5.1 ± 0.4	8.9 ± 1.1	5.6 ± 0.1
		4	4.1 ± 0.7	6.0 ± 0.5	3.9 ± 0.6	5.9 ± 0.9	9.6 ± 1.5	4.9 ± 1.0
27	48	0	3.9 ± 0.4	4.8 ± 0.8	3.9 ± 0.5	4.5 ± 0.6	5.7 ± 0.8	4.0 ± 0.6
		1	6.2 ± 0.5	6.2 ± 0.6	4.1 ± 0.7	12.6 ± 1.5	19.8 ± 2.0	5.4 ± 0.7
		2	17.1 ± 0.4	11.4 ± 2.0	4.0 ± 0.7	24.7 ± 2.0	21.9 ± 4.0	5.3 ± 1.0
		3	13.2 ± 1.5	11.1 ± 1.2	3.8 ± 0.9	43.0 ± 5.0	24.0 ± 3.5	5.9 ± 0.8
		4	13.2 ± 2.0	11.5 ± 1.8	4.0 ± 0.7	63.9 ± 7.0	34.7 ± 5.0	8.0 ± 0.7
28	72	0	3.8 ± 0.5	6.4 ± 1.0	4.0 ± 0.8	3.2 ± 0.7	9.1 ± 2.0	4.9 ± 0.8
		1	3.8 ± 2.0	10.2 ± 1.0	8.1 ± 1.0	2.8 ± 0.7	21.4 ± 4.0	20.1 ± 2.0
		2	3.9 ± 0.4	11.9 ± 2.0	11.1 ± 2.0	3.0 ± 0.4	20.0 ± 3.0	29.5 ± 3.0
		3	3.7 ± 1.0	12.1 ± 1.9	12.9 ± 0.9	2.8 ± 0.6	22.1 ± 2.5	28.5 ± 1.9
		4	3.7 ± 0.8	12.5 ± 2.0	12.9 ± 0.1	2.8 ± 0.5	15.0 ± 1.8	30.5 ± 4.0
28	Saline (Control)	0	4.0 ± 0.7	4.7 ± 0.7	3.9 ± 0.5	4.0 ± 0.6	4.9 ± 0.8	3.8 ± 0.6
		1	3.9 ± 0.6	5.0 ± 0.1	3.6 ± 0.1	3.9 ± 0.5	5.7 ± 1.8	4.9 ± 0.5
		2	3.9 ± 0.8	5.1 ± 0.6	3.7 ± 0.5	4.3 ± 0.4	5.9 ± 1.1	3.9 ± 1.0
		3	4.1 ± 0.7	6.5 ± 1.5	3.8 ± 1.0	4.5 ± 0.4	8.1 ± 0.9	4.1 ± 0.7
		4	4.0 ± 1.0	6.7 ± 0.9	3.9 ± 0.5	5.1 ± 0.8	8.5 ± 0.9	4.0 ± 0.6

FIGURE 5.1 PLASMA STEROID LEVELS IN IMMATURE RATS INJECTED WITH 5 IU PMSG (▨) OR WITH SALINE (□) ON DAY 25 OF LIFE.



The experiment was performed with groups of 4 PMSG-primed rats or the corresponding saline treated control animals. The animals were killed at the times indicated on the abscissa. Plasma samples were collected, extracted with ether and assayed for oestradiol (a), testosterone (b) and progesterone (c) using the specific radioimmunoassay system described in Chapter 3. Results are plotted as means ± SEM (n = 4) *P < 0.05, **P < 0.02, ***P < 0.01; values significantly higher than those obtained from corresponding saline treated control animals.

FIGURE 5.2 EFFECT OF INJECTING SODIUM PENTOBARBITONE AT 14.00 H INTO IMMATURE RATS WHICH HAD BEEN INJECTED WITH PMSG 52 H PREVIOUSLY, ON THE SECRETION OF STEROIDS BY THE OVARIES IN VITRO.



The experiment was performed with 4 groups of ovary which were harvested at times indicated on days 27 and 28, after donors had been primed with 5 iu PMSG on day 25. Ovaries in group 1 were from rats killed between 09.00-10.00 h on day 27 i.e. before the expected LH surge; group 2, from rats killed between 20.00-21.00 h on day 27 i.e. after the endogenous LH surge; group 3, from rats injected with sodium pentobarbitone (Sagatal) 6 to 7 h earlier and the last group, from rats injected with 'Sagatal' 19 to 20 h earlier. The amounts of oestradiol (E), testosterone (T) and progesterone (P) accumulating in the medium during 4 h incubation in hormone free medium (open bars) and in medium containing 1.0 iu/ml HCG (hatched bars) are shown. Results are represented as means ± SEM of 4 incubations. *P < 0.05, **P < 0.02, ***P < 0.01; values significantly higher than those obtained in hormone free medium.

FIGURE 5.3 EFFECT OF PMSG ON FOLLICULAR DEVELOPMENT IN IMMATURE RAT OVARIES.

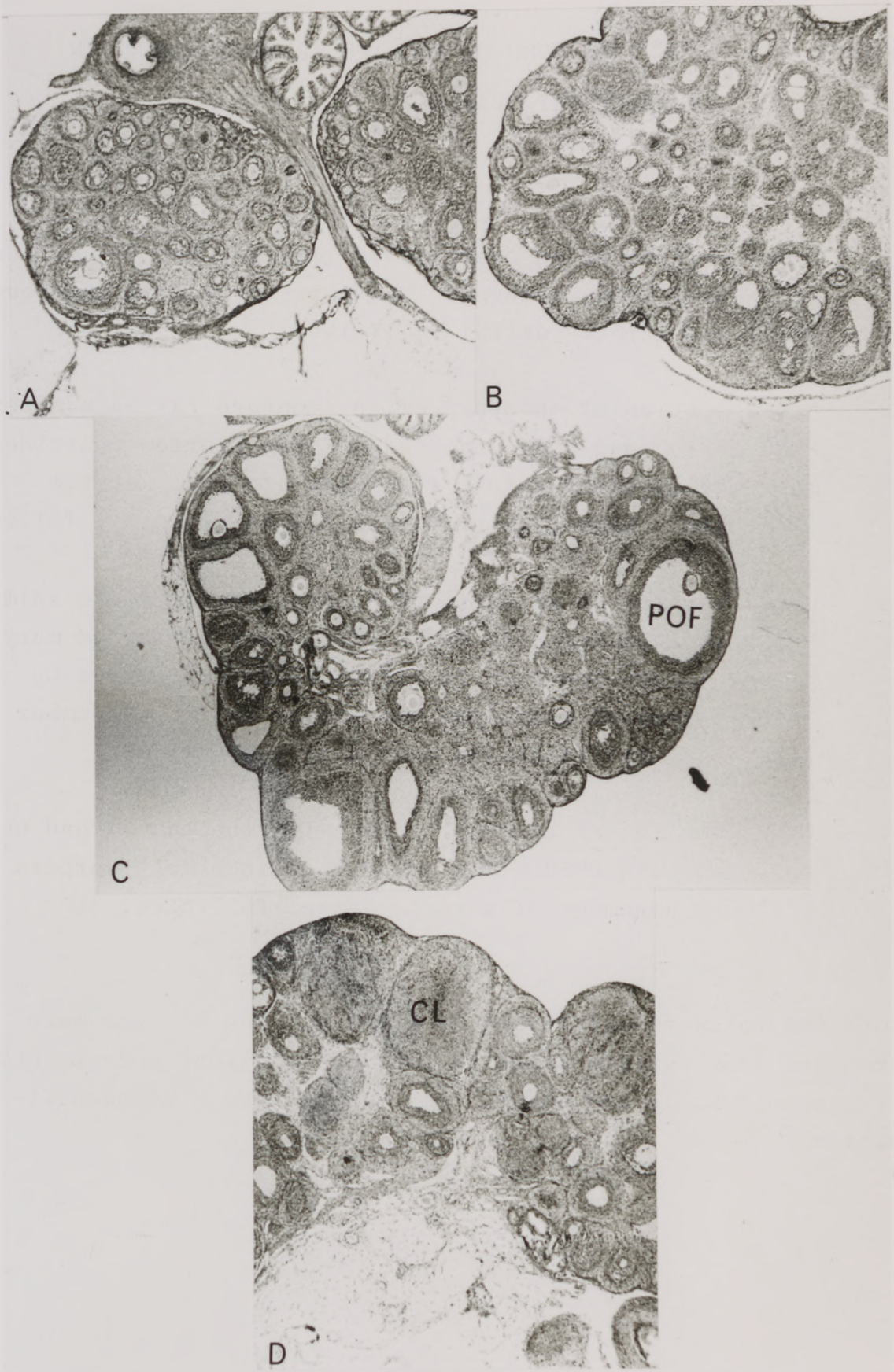
A. Cross section of an ovary of an immature rat 72 h after saline had been injected. The ovary contained numerous small and atretic antral follicles.

B. Cross section of an ovary of an immature rat 24 h after PMSG (5 iu) had been injected. These ovaries contained many medium-sized antral follicles and some atretic follicles. Also present were a few large antral follicles.

C. Cross section of an ovary 48 h after the immature animal had been injected with PMSG. The ovary contained many pre-ovulatory Graafian follicles (POF) surrounded by thick layers of granulosa and thecal cells. A number of atretic antral follicles were also observed.

D. Cross section of an ovary 72 h after the animal had been injected with PMSG. The ovary contained many corpora lutea (CL) and a number of atretic antral follicles.

Note the thick thecal layers in C and D. The section were prepared from ovaries fixed in Bouin's solution, and stained with haematoxylin and eosin. All photographs x 24 magnification.


A
B
POF
C
CL
D

5.3 DISCUSSION

Unless a very large group of animals is available it may be difficult to obtain a sufficient number of animals at the same stage of oestrous cycle on one day. Furthermore the variations which occur in the length of the oestrous cycle have to some extent restricted studies on the endocrine changes which are associated with ovulation in adult rats. As a result, alternative model systems have been developed which can be used to investigate ovarian steroidogenesis. One of these is the immature female rat, in which ovulation can be synchronized by injecting PMSG. With this model, the endocrine changes which occur around the time of ovulation can be studied. The use of such a system can only be useful if there is a good agreement between the results obtained in the PMSG primed rats and those occurring spontaneously in the adults. In the present studies, the effect of injecting PMSG on organ weights and hormone levels in plasma and in incubation media containing the ovaries, both before and after ovulation, have been examined. The results demonstrate that priming 25-day old rats with PMSG (5 iu) resulted in an increase in both uterine and ovarian weights (Table 5.1). The PMSG also caused an increase in steroid secretion (Figure 5.1 and Table 5.3). An interesting aspect of these data is the timing of the changes in organ weights in relation to the expected time of gonadotrophin surge: there is an increase in the uterine weights in the 24 h PMSG-primed animals, indicating that the plasma oestrogen activity is already raised in advance of the expected time of the LH surge.

The effects of PMSG on the plasma steroid levels in immature female rats have been previously reported (Wilson, Horth, Endersby and McDonald, 1974 and Hillensjo et al., 1976). In agreement with these reports, PMSG was found to increase dramatically the concentrations of oestradiol and testosterone in the immature rats 48 h after the PMSG had been injected. In addition, there was a significant increase in plasma progesterone levels in the animals which had been injected with PMSG 72 h previously. At this time, oestradiol levels were minimal and testosterone levels were also lower than 24 h previously. The absolute levels of the measured steroids in the immature rats which had received PMSG 48 h previously were similar to the levels which we found earlier in the adult pro-oestrus rat (Table 3.4). The pattern of changes in the PMSG-injected immature rats in oestradiol levels were at maximum in the 48 h PMSG-primed animals (analogous to pro-oestrus), and declined to a minimum in the 72 h primed animals (oestrus); progesterone levels were low in the 48 h primed animals but were increased substantially during the subsequent 24 h after the expected surge of LH/FSH and ovulation (as evidenced by the presence of ova in the oviducts of the 72 h primed animals, Table 5.2.2). Testosterone levels, like those of oestradiol, were maximum in the 48 h primed animals but unlike oestradiol did not decline drastically during the subsequent 24 h. Dupon and Kim (1973) and the results of Table 3.4 have shown that the concentrations of testosterone are higher on pro-oestrus than on oestrus. This is in agreement with the present findings for the PMSG-primed immature rats in which the plasma testosterone levels in the 48 h primed animals were higher than

those in the 72 h primed animals.

It has been demonstrated from the present studies (Table 5.3) that ovaries from immature rats primed with PMSG for 48 h secrete predominantly oestradiol and testosterone during a 4 h incubation period. This can be compared with the observation that the Graafian follicles of the pro-oestrus rat harvested before the endogenous LH surge and cultured for 4-12 h also secrete principally oestradiol and testosterone (Tsafriri et al., 1973 and Hillensjo et al., 1976). It has also been shown that the addition of HCG to the incubates of ovaries from immature rats treated 48 h previously with PMSG, caused a marked increase in the secretion of oestradiol and testosterone. Again this is similar to the results obtained using individually cultured Graafian follicles isolated from PMSG-primed ovaries from immature rats (Hillensjo et al., 1976). Similarly, the 72 h PMSG-primed ovaries and the isolated follicles harvested after the expected LH surge, secreted mostly progesterone without a further stimulation of testosterone or oestradiol when HCG was added. However, when the endogenous LH surge was blocked (by administering sodium pentobarbitone at 14.00 h of 'pro-oestrus'), the 72 h primed ovaries secreted predominantly oestradiol and testosterone and only small amounts of progesterone. These rats had no ova in their oviducts. Thus the profile of steroids secreted at this time resembled that in the 48 h primed animals (Fig. 5.2). This supports the hypothesis that the LH/FSH surge, and the events attendant upon it, are responsible for inducing the inhibition of oestrogen synthesis which occurs between pro-oestrus and oestrus.

It is noteworthy that the ovaries from the 48 h PMSG-primed rats, as shown in histological studies (Fig. 5.3c), contained many pre-ovulatory Graafian follicles. The follicular development of the (48 h) PMSG-primed rats described here, was similar to that observed after 48 h of FSH treatment to oestrogen primed hypophysectomized rats and to that of pre-ovulatory follicles in adult animals on the morning of pro-oestrus (Erickson and Hsueh, 1978). Other investigations have shown that granulosa cell aromatase activity attains maximal levels in pre-ovulatory follicles during the same period (i.e. the morning of pro-oestrus) in cyclic rats (Hillier et al., 1980a). From these observations it would seem that the activation or induction of granulosa cell aromatase activity is a function of FSH acting during the course of normal pre-ovulatory follicular maturation (Hillier et al., 1980b).

In summary the present results are consistent with the reports that the pre-ovulatory Graafian follicles harvested on the morning of pro-oestrus of adult rat and ovaries taken from immature rat primed with PMSG for 48 h are similar both in their secretion of steroids in vitro and in their response(s) to LH/HCG (Tsafriri et al., 1973; Hillensjo et al., 1976; Ahren et al., 1979).

The physiological gonadotrophin stimulation would seem to have a dual effect: after a transitory stimulation, it inhibits progesterone secretion by the pre-ovulatory follicles (Hillensjo et al., 1976). Some evidence for the mechanisms which might explain this LH-induced decline in androgens and oestrogens around the time ovulation is discussed in Chapter 9.

CHAPTER 6

STUDIES ON THE ROLE OF HCG-STIMULATED PROTEIN SYNTHESIS IN THE OESTRADIOL SECRETED BY THE OVARIES OF PMSG-PRIMED IMMATURE RATS IN RESPONSE TO HCG

6.1 INTRODUCTION

It has been shown that while testosterone is secreted by the thecal cells of the rat ovary, oestradiol is secreted by the granulosa cells (Fortune and Armstrong, 1978). In the preceding chapter it was shown that HCG (or LH) would stimulate the secretion of both testosterone and oestradiol from pre-ovulatory ovaries of PMSG-primed rats. The oestradiol secreted under these circumstances in response to HCG however, may be principally due to an enchanced secretion of testosterone and the production of increased amounts of substrate for aromatization. It was also shown (in Chapter 4) that oLH and HCG had relatively little effect on oestrogen secretion by isolated granulosa cells, an observation which supports the preceding hypothesis.

However, the available evidence suggests that the effect of LH on follicular steroidogenesis is mediated by the synthesis of specific protein(s). This suggestion is based on the effects of inhibitors of protein and RNA synthesis on steroid production (Lieberman, Barnea, Bauminger, Tsafriri, Collins and Lindner, 1975; Younglai, 1975).

This possibility has been investigated further by incubating the ovaries from PMSG-primed immature rats in the presence of varying concentrations of testosterone, and in the presence or absence of HCG and/or inhibitors of protein synthesis

(puromycin or cycloheximide).

6.2 EXPERIMENTAL PROCEDURE

Immature female rats were injected with 5 iu PMSG on the morning of day 26 of age and killed 48 h later. The ovaries were removed and placed individually in culture dishes containing varying concentrations of testosterone (1×10^{-10} M to 1×10^{-6} M), puromycin* (50 µg/ml), cycloheximide @5.0 µg/ml), and/or HCG (1.0 iu/ml) and incubated for 4 h in KRB (supplemented with glucose and glutamine) as described previously. At the end of the incubation period, aliquots of medium were withdrawn and frozen for subsequent steroid assay using the specific radioimmunoassay described in Chapter 3.

* Puromycin was later replaced by cycloheximide because puromycin has been shown to be relatively non specific, inhibiting both RNA and protein synthesis (Ennis and Lubin, 1964).

6.3 RESULTS

Effect of different concentrations of testosterone on ovarian oestradiol production

The effect of increasing concentrations of exogenous testosterone on oestradiol secretion by the ovaries is summarized in Table 6.1. There was a positive, linear correlation between the concentration of testosterone (1×10^{-10}M to 1×10^{-6}M) added to the ovarian incubates at the beginning of incubation and the oestradiol accumulating in the medium at the end of the 4 h incubation ($R^2 = 0.949$). Testosterone enhanced oestradiol secretion at 1×10^{-9}M but at this level it was not

significantly different from the secretion of oestradiol by the control ovaries. Maximal stimulation (up to 2.1 ± 0.28 pmoles oestradiol/h/ovary was achieved with 1 x 10^{-7}M testosterone, with no subsequent significant rise at the higher level of added steroid (1 x 10^{-6}M).

Effect of increasing concentrations of cycloheximide on HCG-stimulated ovarian oestradiol and testosterone production

Concentrations of cycloheximide ranging from 5.0 to 50.0 µg/ml were added to the incubates of ovaries from immature rats primed with PMSG in the presence or absence of HCG (1.0 iu/ml). The dose-response curves obtained are shown in Fig. 6.1. The minimum effective dose of cycloheximide which inhibited the HCG enhanced oestradiol and testosterone secretion was 10 µg/ml while maximal inhibition was achieved with 25 µg/ml medium.

Effect of cycloheximide (25 µg/ml) on oestradiol accumulating in the medium containing ovaries of immature rats primed with PMSG in the presence or absence of HCG (1.0 iu/ml) and/or testosterone (1 x 10^{-7}M)

HCG (1.0 iu/ml) stimulated the secretion of oestradiol. The concentrations of oestradiol in the incubation media after 4 h in this series of experiments, were 1.20 ± 0.11 pmole/ml and 3.52 ± 0.13 pmole/ml for the ovaries without added HCG and with added HCG respectively ($P < 0.01$) (Table 6.2).

The addition of testosterone (1 x 10^{-7}M) to the medium containing ovaries from immature rats which had been injected with saline (controls) produced negligible amounts of oestradiol (0.31 ± 0.05 pmole/ml): Table 6.2. When both testosterone

(1×10^{-7}M) and HCG (1.0 iu/ml) were added to the culture dishes containing the PMSG-primed ovaries, their combined effect on oestradiol secretion was greater than that of either hormone alone, being significantly greater than HCG action alone ($P < 0.05$) but not significantly greater than testosterone alone.

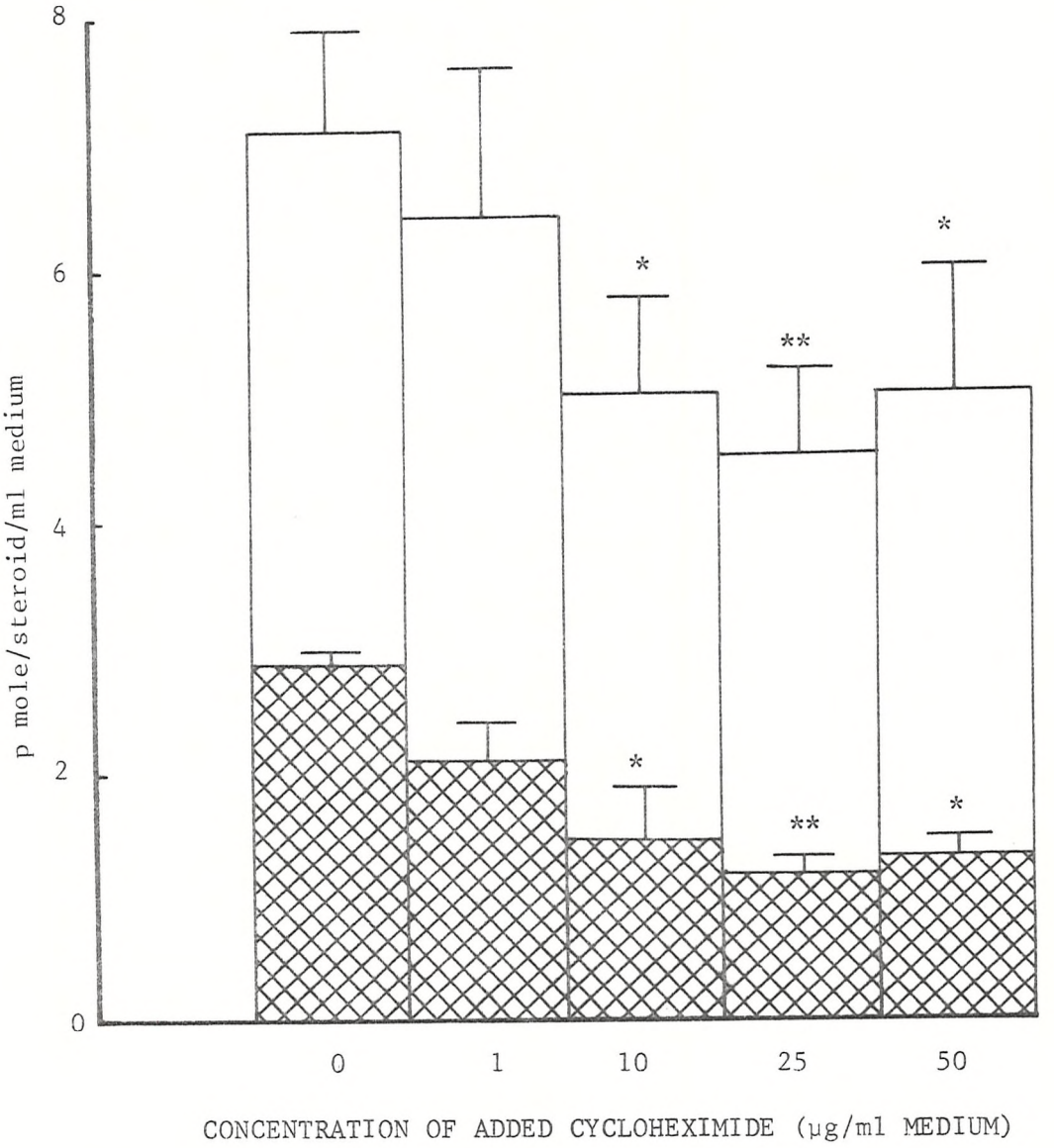
Cycloheximide (25 µg/ml) significantly inhibited the HCG-stimulated oestradiol secretion. The oestradiol secreted in the presence of HCG was reduced from 3.52 $\pm$ 0.13 to 1.42 $\pm$ 0.13 pmoles/ml by the addition of cycloheximide ($P < 0.02$). Oestradiol levels in the media, in the presence of 1×10^{-7}M testosterone, were reduced from 6.0 $\pm$ 0.8 to 4.95 $\pm$ 0.83 pmoles/ml, but this suppression was not significant. The addition of cycloheximide in the presence of both HCG and testosterone reduced the oestradiol concentration in the medium from 8.2 $\pm$ 1.6 to 5.2 $\pm$ 1.0 pmoles/ml, but this reduction was not significant. These results are summarized in Figure 6.2.

Table 6.1 Oestradiol secreted by the ovaries of PMSG-primed immature rats in the presence or exogenous testosterone.

Amount of testosterone added to the incubation medium	oestradiol concentration (pmole/ml medium)
0 (controls)	2.0 ± 0.3
1×10^{-10}M	2.6 ± 0.5
1×10^{-9}M	3.0 ± 0.5
1×10^{-8}M	5.1 ± 0.8*
1×10^{-7}M	8.5 ± 1.0**
1×10^{-6}M	8.4 ± 0.9**

The experiment was performed with ovaries harvested from 28 day old rats previously primed with PMSG (5 iu) for 48 h. The ovaries were incubated in hormone free medium (control) or in medium containing increasing concentrations of testosterone (1×10^{-10}M to 1×10^{-6}M). The oestratiol accumulating during the 4 h incubation was assayed by radioimmunoassay as previously described. Results are shown as means ± SEM of four incubations. *$P < 0.02$, ** $P < 0.01$: P values for oestradiol concentrations significantly higher than those obtained in the control medium.

FIGURE 6.1 EFFECT OF INCREASING CONCENTRATIONS OF CYCLOHEXIMIDE ON HCG STIMULATED OESTRADIOL (▨) AND TESTOSTERONE (□) SECRETION BY PMSG PRIMED IMMATURE RAT OVARIES.



The experiment was performed with ovaries harvested from 28 day animals which had been primed with PMSG (5 iu) for 48 h. The ovaries were incubated in medium containing HCG (1.0 iu/ml) and no cycloheximide (0, control) or in medium containing HCG and increasing concentrations of cycloheximide (1.0 - 50 µg/ml). Oestradiol and testosterone levels accumulating in the medium during the 4 h incubation, were assayed. Results are shown as means ± SEM of four incubations. *P < 0.05; **P < 0.02. P values significantly lower than those obtained in the control medium.

Table 6.2 Effect of cycloheximide (25 µg/ml) on oestradiol secretion by PMSG-primed ovaries in the presence or absence of HCG (1.0 iu/ml) and/or testosterone (1×10^{-7} M).

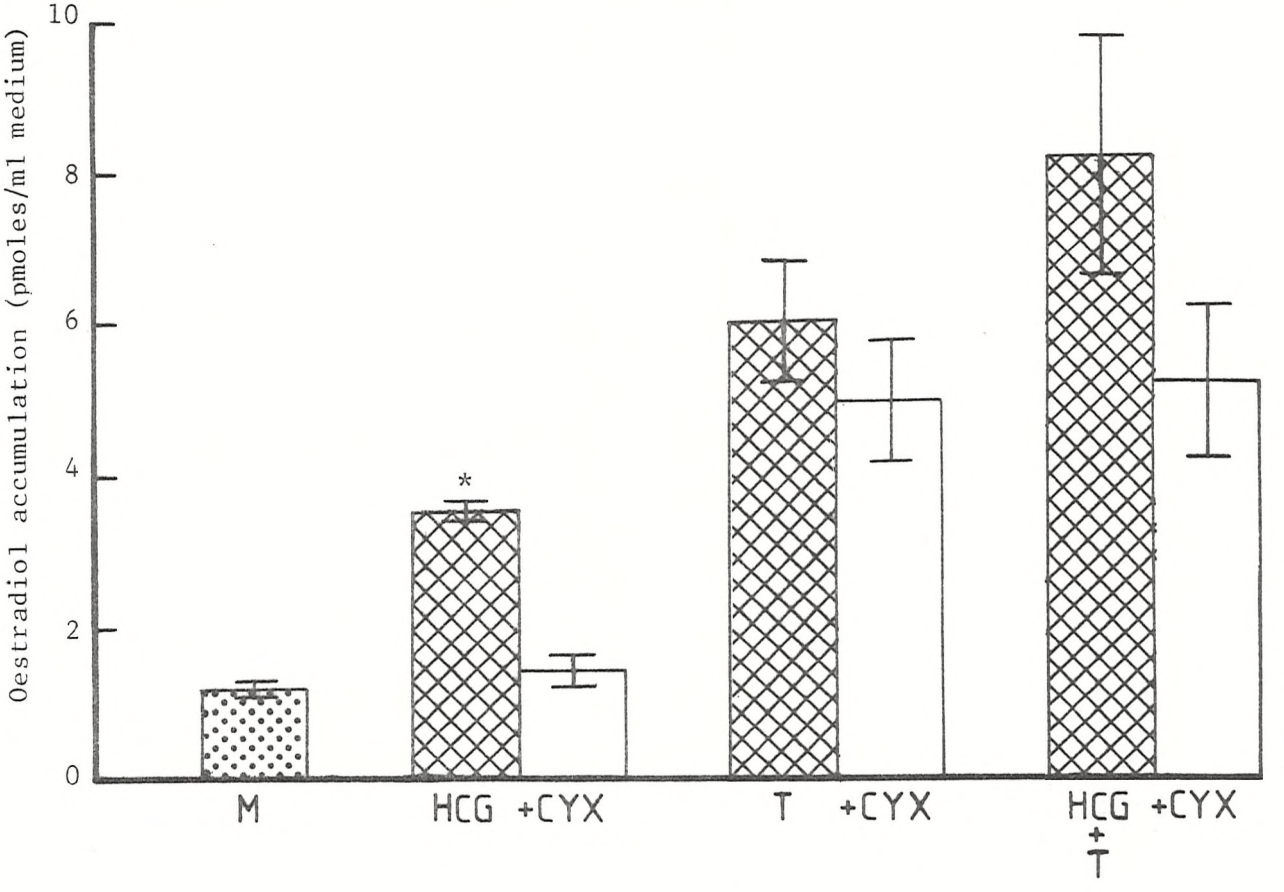
	Addition to the incubation medium	Oestradiol concentration (pmole/ml medium)
1	PMSG-primed ovaries alone (control)	1.20 ± 0.11^{e}
2	Cycloheximide	0.81 ± 0.12^{f}
3	HCG	3.52 ± 0.13^{d}
4	HCG + cycloheximide	1.42 ± 0.20^{e}
5	Testosterone	6.02 ± 0.81^{ac}
6	Testosterone + cyclo-heximide	4.95 ± 0.83^{c}
7	HCG + Testosterone	8.22 ± 1.62^{a}
8	HCG + Testosterone + cycloheximide	5.21 ± 1.10^{ac}
9	Ovaries from rats treated with saline + Testosterone	0.31 ± 0.05^{g}
10	Ovaries from rats treated with saline + Testosterone + cycloheximide	0.30 ± 0.06^{g}

Groups 1-8 utilized ovaries from 28 day old rats primed with PMSG (5 iu) 48h previously; groups 9-10 contained ovaries from comparable saline treated controls. The ovaries were incubated in medium containing the various additions as shown in the table. Oestradiol accumulating during the 4 h incubation was assayed by radioimmunoassay. Results are shown as means ± SEM of four incubations.

The data were examined by analysis of variance and the means compared by the Duncan's multiple Range test.

$^{a-g}$mean values without a common superscript are significantly different ($p < 0.05$).

FIGURE 6.2 EFFECT OF CYCLOHEXIMIDE ON OESTRADIOL SECRETION BY PMSG-PRIMED RAT OVARIES



The experiment was performed with PMSG-primed ovaries collected from 28 day old animals as previously described. They were incubated in medium as described previously. Oestradiol accumulating during the 4 h incubation period in hormone free medium (M, stippled bar) or medium containing HCG, Testosterone (T) or HCG plus T (cross hatched bars) is shown. Incubation dishes containing HCG plus cycloheximide (CYX), T plus CYX, or the combined hormones plus CYX, are represented by the open bars. Results are shown as means ± SEM of four incubations. *P < 0.02; values significantly higher than those obtained in the presence of cycloheximide.

6.4 DISCUSSION

The results so far indicate that the principal action of HCG may be to raise the endogenous levels of substrate (testosterone) and this may in turn increase oestradiol accumulating in the medium. This is supported by the observation that increasing concentrations of exogenous testosterone increase the amount of oestradiol which accumulate in the medium. These results are compatible with the reports of Fortune and Armstrong (1978) that the addition of testosterone to the culture medium containing granulosa cells isolated from the ovaries of rats primed with PMSG caused a dramatic increase in oestradiol secretion. (Although the isolated thecal cells in this preparation did not show a similarly enhanced secretion of oestradiol). However, in the present experiment, the ovaries from saline treated animals did not secrete oestradiol during 4 h incubation in the presence of exogenous testosterone. A possible explanation for this difference may be that prior exposure of rats to PMSG *in vivo* (for 2 days), resulted in a stimulation of aromatase activity in granulosa cells (Fortune and Armstrong, 1978; Suzuki, Kawakura and Tamaoki, 1978). The raised amounts of active enzyme probably persisted during the 4 h incubation. Since the PMSG-primed ovaries possess an activated aromatase system, the supply of aromatizable androgen (testosterone) leads to the enhanced oestradiol production which we observed. Such a conclusion is consistent with previous observations that conversion of exogenous testosterone to oestradiol by granulosa cells of cycling rats is correlated with aromatase activity (Hillier, Van den Boogaard, Reichert, Van Hall, 1980a).

No evidence can be obtained from the present studies which confirm or substantiate the evidence about which cell type carries out the final enzymatic conversion (aromatization) of testosterone to oestradiol. However, thecal cells contain the LH receptors necessary for the response to LH/HCG (Richards and Midgley, 1974). Moreover, isolated thecal cells produce androgens when stimulated by endogenous (Makris and Ryan, 1975) or exogenous (Fortune and Armstrong, 1979) LH. In the rodent ovary, it seems clear that the granulosa cells in developing preovulatory follicles possess the capacity to aromatize the androgens produced by the LH-stimulated thecal tissue. It therefore seems reasonable to suggest that the oestradiol secretion which we observed occurred through the co-operation of the two cell types, the thecal and the granulosa cells.

It has been widely reported that protein synthesis may play a role in the LH stimulation of steroidogenesis. Savard, Marsh and Rice (1965) indicated that both puromycin and actinomycin D prevent the action of LH in stimulating steroidogenesis in bovine corpus luteum. Younglai (1975) also reported that puromycin and cycloheximide can inhibit the LH-stimulated steroidogenesis by isolated ovarian follicles. Addition of cycloheximide to rat Leydig-cell suspensions inhibits LH stimulated testosterone secretion (Cooke, Janszen, Clotscher and Van der Molen, 1975). Our results are similar despite the differences in experimental models.

As may be seen in Fig. 6.2, the presence of cycloheximide suppressed HCG-stimulated oestradiol secretion. This result suggests that protein synthesis is required for HCG-induced oestradiol production. However, it cannot be concluded from

the present studies whether the dose of cycloheximide used in suppressing HCG-induced steroidogenesis caused a parallel inhibition of protein synthesis in the ovary. Evidence is presented in Chapter 7 which suggest that the effects of cycloheximide on the ovary are due principally to their effects on protein synthesis. The inhibitory effect of cycloheximide on steroid secretion by the control ovary in the absence of exogenous HCG, may be due to the inhibition of the action of endogenous gonadotrophin (LH), hence reducing the supply of available testosterone for subsequent aromatization within the follicles.

However, it appears from the present studies that testosterone stimulated oestradiol production does not involve the de novo synthesis of protein(s) of short half life since the production of oestradiol by ovaries in the presence of testosterone was not significantly affected by cycloheximide. A likely explanation for the different actions of the two hormones (HCG and testosterone) might be that the effect of the HCG (mediated through de novo protein synthesis, Lindner et al., 1974) is at site(s) removed from the aromatization stage where testosterone (endogenous and exogenous) is converted to oestradiol.

In conclusion, it is apparent from the present study that a cycloheximide-sensitive product is required for the HCG-induced increase of oestradiol production. Its site of action is probably before the aromatization stage. However, its precise mode of action remains to be elucidated.

CHAPTER 7

STUDIES ON THE EFFECT OF CYCLOHEXIMIDE ON HCG-INDUCED STEROIDOGENESIS AND PROTEIN SYNTHESIS BY PMSG-PRIMED OVARIES IN VITRO

7.1 INTRODUCTION

Studies from the preceding two chapters have shown that HCG stimulates steroid production *in vitro* from ovaries of the immature rat which has been primed with PMSG. It was shown that protein synthesis may play a role in this HCG-induced steroidogenesis. Addition of an inhibitor of protein synthesis, cycloheximide (10 µg/ml), to ovarian incubates, significantly inhibited the HCG-induced oestradiol and testosterone secretion (Fig. 6.1). These results are in agreement with earlier reports in which Graafian follicles were explanted from rat ovaries in the morning of pro-oestrus (Tsafriri, Lieberman, Barnes, Bauminger and Lindner, 1973; Lindner, Tsafriri, Lieberman, Zor, Koch, Bauminger and Barnes, 1974), and in which the changes in steroidogenesis induced by LH/HCG were inhibited by the addition of cycloheximide. Comparable results have been reported for other steroid-synthesizing tissues, e.g. rat testicular Leydig cells (Moyle, Moudgal and Greep, 1971; Janszen, Cooke and Van der Molen, 1976); adrenal cortex (Garren, May and Davis, 1965; Lowry and McMartin, 1974).

To obtain more information about the role of the protein involved in the HCG-induced steroidogenesis, we have now studied the effect of cycloheximide on HCG-induced protein synthesis and steroid secretion. The aim was to establish that the effect of the cycloheximide on the HCG-stimulated steroidogenesis was due to its effect on protein synthesis and not due

to a non specific 'toxic' effect. This assumption would be justified if inhibition of protein synthesis and steroids production were parallel with different doses of cycloheximide. The results obtained are discussed in relation to the possible role of HCG-induced protein(s) in the regulation of steroidogenesis.

7.2 METHODS

28-day old PMSG-primed rats were killed 48 h after the gonadotrophin treatment as previously described. The ovaries were removed, cleared of connective tissue, and the wet weight was recorded. Each ovary was incubated in KRB (supplemented with glucose). HCG (1.0 iu/ml), cycloheximide (5 or 25 µg/ml) and ^{3}H-Leucine (3 µCi/ml) were added to the medium in the appropriate treatment groups at the start of the incubation (as shown in Table 7.2). The final volume of the incubation medium was 1 ml.

At the end of the incubation the ovaries were quickly removed into 25 ml homogenizing tubes containing 5 ml 10% TCA/100 mM Leucine to stop further uptake of ^{3}H-Leucine. Aliquots of the incubation media were stored at -20^{o}C for subsequent determination of steroid content. The ovaries were homogenized, transferred to a 12 ml centrifuge tubes and centrifuged at room temperature (2 min at 1000 g). The supernatant was discarded and the pellet was resuspended in a further 5 ml 10% TCA/100 mM Leucine, homogenized and centrifuged. This resuspension was repeated 2 times. The pellet was resuspended (twice) in 5 ml ether to remove TCA and centrifuged at room temperature (2 min at 1000 g). The final pellet was dissolved at 60^{o}C in 1 ml of

0.25 M KOH. 0.75 ml of the dissolved pellet was transferred to a scintillation vial, 250 µl of water and 10 ml Trioscint added. To the mixture, 50 µl glacial acetic acid was added (to acidify the scintillant to prevent phosphorescence) and radioactivity counted as previously described (Chapter 3). The corrected counts obtained were taken as a measure of total ^{3}H-Leucine uptake.

A 20 µl portion of the dissolved pellet was used for protein determination according to the method originally described by Lowry et al,, (1951) using bovine serum albumin (BSA) as the standard.

Radioimmunoassay

Aliquots of the medium collected at the end of the 4 h incubation for steroids assay were first extracted with ether (this ensured that no ^{3}H-Leucine remained in the extract). The dried ethereal extracts were then assayed for oestradiol, testosterone and progesterone using the specific radioimmunoassay system already described.

7.3 RESULTS

Effect of HCG on the incorporation of ^{3}H-Leucine into protein by PMSG-primed ovaries

Table 7.1 shows the incorporation of ^{3}H-Leucine into ovarian proteins as a function of incubation time, in the presence or absence of HCG. The addition of HCG to the incubation did not significantly increase the overall rate of incorporation of ^{3}H-Leucine into protein when compared with ovaries incubated without HCG. Analysis of the two slopes

(analysis of co-variance) revealed that the two lines were not significantly different.

Inhibition of protein synthesis by cycloheximide (5.0 or 25 µg/ml) in PMSG-primed ovaries

Incubation of PMSG-primed ovaries in medium containing HCG (1.0 iu/ml) and cycloheximide (5 µg/ml) reduced the incorporation of ^{3}H-Leucine into TCA precipitable protein by 94%. There was no further suppression of protein synthesis when a higher concentration of cycloheximide (25 µg/ml) was added to the medium. These results are compared in Table 7.2. The inhibitory action of cycloheximide was unaffected by the presence or absence of HCG in the incubation medium.

Effect of cycloheximide on HCG-induced steroid production by PMSG-primed ovaries in vitro

Incubation of PMSG-primed ovaries with HCG (1.0 iu/ml) resulted, at the end of 4 h, in an increased accumulation of oestradiol (E), testosterone (T), and progesterone (P) in the medium. The levels of E, T and P in the medium in the absence of HCG were 1.25 $\pm$ 0.27; 1.69 $\pm$ 0.20 and 1.07 $\pm$ 0.21 pmoles/ml respectively and these levels were raised to 3.87 $\pm$ 0.44 ($P < 0.02$); 7.43 $\pm$ 0.73($p < 0.02$) and 6.41 $\pm$ 0.51 ($P < 0.02$) p moles/ml respectively by the addition of HCG (Table 7.2).

Cycloheximide (5 µg/ml) partially suppressed the HCG-induced secretion of E, T and P by 36.2%, 34.3% and 55.7% respectively. At the higher concentration of cycloheximide used (25 µg/ml), the inhibitor abolished the HCG-induced increase in steroid levels. These results are compared in Table 7.2 and Figure 7.1.

Table 7.1 Time course of the incorporation of ^{3}H-Leucine (3 µCi/ml) into TCA precipitable protein by PMSG-primed ovaries incubated with or without HCG (1.0 iu/ml).

Incubation duration (h)	TCA precipitable proteins (dpm ^{3}H/mg protein x 10^{4})	
	with HCG	without HCG (Control)
1	8.0 ± 1.2	10.1 ± 1.1
2	20.1 ± 2.1	20.0 ± 2.8
3	50.5 ± 6.0	44.6 ± 3.8
4	68.8 ± 7.3	72.2 ± 7.5
r^{1}	0.91	0.95
slope[2]	21.33	21.85

The experiment was performed with ovaries harvested from 28 days old rats previously primed with PMSG (5 iu) for 48 h. The ovaries were incubated for different time-periods up to 4 h with ^{3}H-Leucine (3 µCi/ml) and with or without HCG (1.0 iu/ml). At the end of each incubation period, the ovaries were homogenized for the subsequent determination of ^{3}H-Leucine incorporated into TCA precipitable protein. For further details see 'Methods'.

[1]The correlation coefficient (r) was calculated from the calculated line of closest fit to the data, using an analysis of least squares, and a Hewlett-Packard calculator.

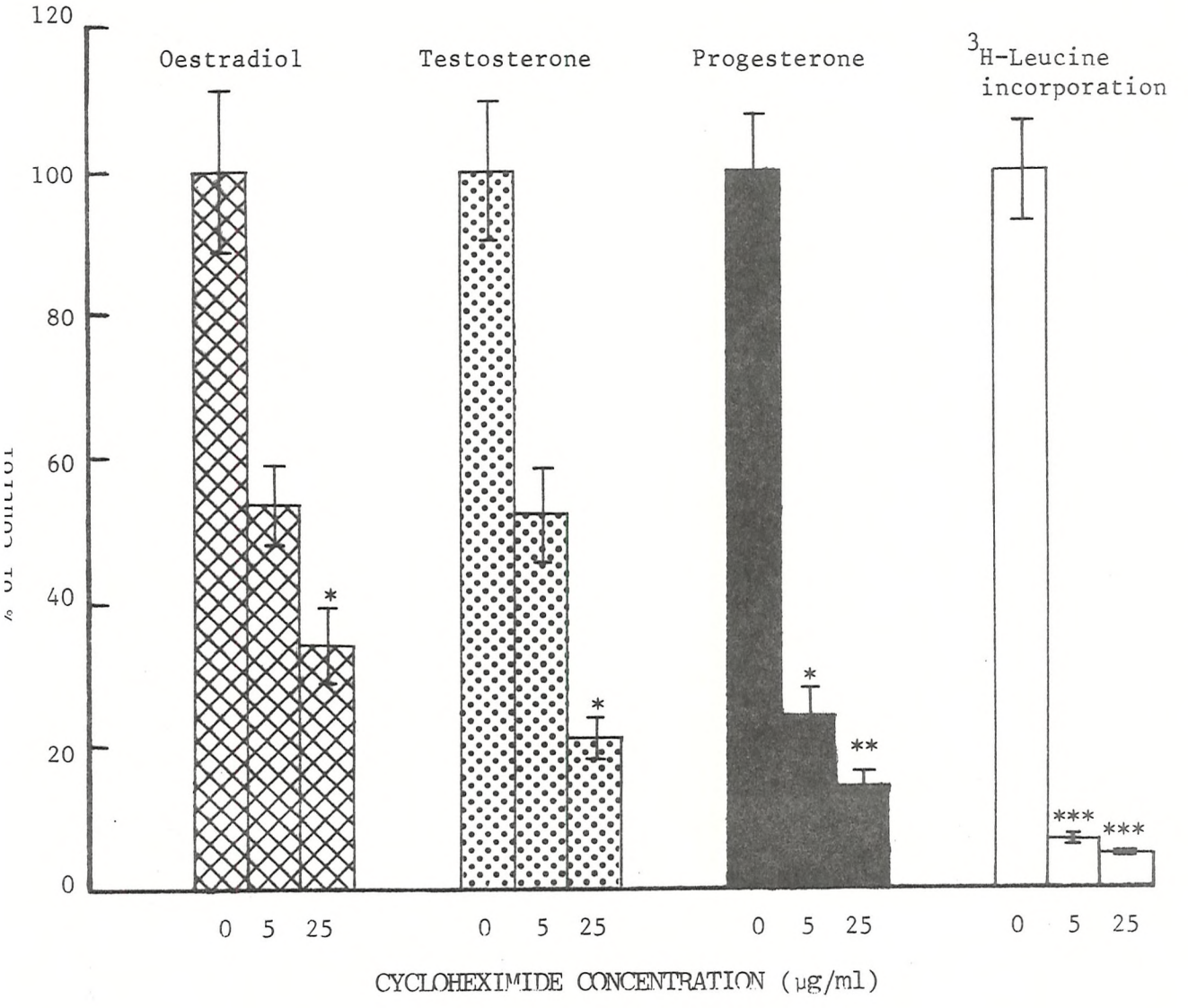
[2]The slopes were compared by analysis of co-variance using a Hewlett-Packard digital calculator. There was no significant difference between the slopes of the lines with or without the addition of HCG.

Table 7.2 Effect of cycloheximide (0 - 25 µg/ml) on HCG (1.0 iu/ml) induced steroid production and the incorporation of ^{3}H-Leucine (3 µCi/ml) into TCA precipitable proteins.

Medium additives	Steroid accumulation in the medium (pmole/ml medium)			dpm ^{3}H-Leucine/mg protein
	oestradiol	Testosterone	Progesterone	
^{3}H-Leucine (3 µCi/ml)	1.25 ± 0.27**	1.69 ± 0.20**	1.07 ± 0.21**	338.4 ± 45.6
HCG (1.0 iu/ml) + ^{3}H-Leucine (3µCi/ml) (control)	3.87 ± 0.44	7.43 ± 0.73	6.41 ± 0.51	363.8 ± 25.5
HCG (1.0 iu/ml) + ^{3}H-Leucine (3 µCi/ml) + 5 µg/ml cycloheximide	2.47 ± 0.21	4.88 ± 0.48	2.84 ± 0.32*	23.29 ± 2.76***
HCG (1.0 iu/ml) + ^{3}H-Leucine (3 µCi/ml) + 25 µg/ml cycloheximide	1.26 ± 0.21**	1.55 ± 0.22**	0.90 ± 0.15***	15.59 ± 1.13***

48h PMSG-primed ovaries of immature rats were incubated in Kreb's buffer, pH 7.4, supplemented with glucose and glutamine. HCG, cycloheximide and ^{3}H-Leucine were added at the start of the incubation. Steroid content in the medium and the incorporation of ^{3}H-Leucine into protein were measured as described in the methods' section. Results are expressed as means ± SEM of 4 incubations. *P < 0.05, **P < 0.01, ***P < 0.001: values significantly lower than those obtained in the medium containing HCG plus ^{3}H-Leucine (control).

FIG. 7.1 EFFECT OF AN INHIBITOR OF PROTEIN SYNTHESIS (CYCLOHEXIMIDE) ON THE HCG-INDUCED STEROID SECRETION AND THE INCORPORATION OF ^{3}H-LEUCINE INTO PROTEINS, BY THE OVARIES OF IMMATURE RATS PRIMED WITH PMSG.



Ovaries of rats which had been injected with 5 iu PMSG 48 h previously were incubated for 4 h as described previously (Chap. 2.3). HCG (1.0 iu/ml), ^{3}H-Leucine (3 µCi/ml) and cycloheximide (0, 5 and 25 µg/ml) were added to the medium at the start of the incubation. Oestradiol (⊠), testosterone (▩) and progesterone (■) accumulating in the medium or ^{3}H-Leucine incorporation into proteins (□) were determined as described in the methods section. Results are expressed as % of the control values (means ± SEM of four incubation dishes). *P < 0.05; **P < 0.01; ***P < 0.001; values significantly lower than those obtained in the absence of cycloheximide.

7.4 DISCUSSION

In the present study it has been demonstrated that the HCG induced steroid secretion by incubates of PMSG-primed rats' ovaries was not accompanied by a detectable increase in the overall rate of incorporation of ^{3}H-Leucine into TCA precipitable ovarian protein during the 4 h incubation. Inhibition (by 94%) of protein synthesis by cycloheximide (5 µg/ml) suppressed the HCG-induced steroid secretion, although at this concentration of cycloheximide the levels of the 3 steroids secreted were still higher than the levels obtained in the absence of HCG (basal levels). However when 25 µg/ml of cycloheximide was used, the HCG induced steroid secretion was abolished. Since ovarian protein synthesis was maximaly inhibited with 5 µg/ml cycloheximide, while steroids secretion were not, it seems superficially unlikely that the increased steroid secretion due to HCG was due to de novo synthesis of protein as has been widely reported (Tsafriri et al., 1973; Lindner et al., 1974; Lieberman et al., 1975).

The possibility that cycloheximide does abolish the HCG-induced steroidogenesis, but enhances secretion of preformed steroid into the medium is a possible explanation for the present results, but this is discounted by our earlier observation that the medium/tissue ratio of the steroids does not change with the addition of 5 µg/ml cycloheximide (Table 3.5). The possibility that 25 µg/ml cycloheximide has no effect on the HCG stimulation of steroidogenesis but may have an inhibitory effect on steroid secretion is again discounted by the fact that medium/tissue ratio of the steroids were not altered when ovaries were incubated with or without cycloheximide. We are

left with two possibilities to account for our present results.

(a) Although cycloheximide (5 µg/ml) may inhibit about 94% of cytosolic protein synthesis, a protein factor present at the 6% level which is involved in steroidogenis may still be at a high enough concentration to permit the enhanced steroid secretion we observed. This explanation seems attractive in the light of 25 µg/ml cycloheximide further reducing steroid secretion, at which concentration of cycloheximide 96% of protein synthesis was inhibited. The difference between 94% and 96% may not be detectable with ^{3}H-Leucine uptake but a suppression from 6% to 4% level of an obligatory protein may significantly effect steroidogenesis.

(b) It has been shown that hormones frequently act by stimulating the adenylate cyclase, which in turn leads to a rise in intracellular c'AMP (Marsh et al., 1966; Rasmussen, 1974). The role of c'AMP in regulating phosphorylation/dephosphorylation of regulatory proteins has been well documented (Newsholme and Start, 1973). Our present results are consistent with the possibility that steroidogenesis may be modulated by changes in intracellular c'AMP levels. Thus, under conditions where protein synthesis is blocked, protein kinase and/or phosphorylase enzymes which are directly 'sensitive' to c'AMP can still exert their regulatory effect on steroidogenesis. However, as the enzymes are gradually catabolised, and are not replaced by de novo synthesized enzymes, so modulation of steroidogenesis is curtailed. It is perhaps more difficult to fit this last model around the data obtained for 25 µg/ml cycloheximide since theoretically, in this system, it is the

degree of catabolism which reduces steroidogenesis. It cannot be ruled out that factors blocking protein synthesis may also modulate protein catabolism by a number of mechanisms. Thus any one or a combination of above explanations may fit into our results.

However, the above models do not account for the differential inhibitory action of cycloheximide on the three ovarian steroids measured, or on the residual protein synthesis observed even in the presence of maximum dose of the inhibitor. These observations are not totally surprising since it is more than likely that HCG may be activating more than one regulatory protein in the steroidogenic pathway. Alternatively, the differential inhibition may be attributed to the specific effect of cycloheximide rather than a generalized non-specific 'toxic' effect (Lindner et al., 1974). Evidence for the specificity of the inhibitory site of cycloheximide in the steroid biosynthetic route, has been provided by Davis and Garren (1968) from which it was shown that cycloheximide inhibited the Adrenocorticotrophin (ACTH) response by preventing the conversion of cholesterol to pregnenolone in adrenal cortical cells. (It should be noted however, that cycloheximide does not itself block the enzyme but will alter the rate of synthesis of the enzyme, or another regulatory protein(s) which affects the enzyme). Since the conversion of pregnenolone to corticosterone was unaffected by cycloheximide, it was concluded that the locus of action of the inhibitor was prior to pregnenolone in the biosynthetic pathway (Garren and Davis, 1968). This conclusion favours our present results: when inhibition occurs, only a small amount of pregnenolone may be available (as substrate) so that progesterone synthesis

drastically falls. Testosterone and oestradiol secretion may depend upon a nascent pool of precursors, the concentration(s) of which may not instantaneously be affected by the lower concentration of cycloheximide. Our present results also showed that the suppression of protein synthesis never reached 100%, and that the residual synthesis (4% of control level) may have included mitochondrial proteins which are insensitive to cycloheximide (Janszen et al., 1976).

To summarize, our results are in accord with the reports of other workers (Tsafriri, et al., 1973; Lindner et al., 1974; Schulster et al.1976; Lieberman et al., 1975) that protein synthesis and gonadotrophin-induced steroidogenesis are both suppressed by cycloheximide, implicating a direct involvement of a labile protein in mediating the HCG induced response. However, our results argue against de novo synthesis of protein as the only pre-requisite for stimulation of steroidogenesis by trophic hormone. It is difficult with the available data to conclude that involvement of HCG-induced protein is obligatory in the stimulation of steroid production.

More information about the mechanisms regulating the ovarian protein(s) synthesized in response to HCG could perhaps be obtained by monitoring the conversion of [^{14}C] - cholesterol to [^{14}C] - progesterone, [^{14}C] - testosterone or [^{14}C] - oestradiol in the presence or absence of HCG and/or cycloheximide. Alternatively, experiments may be designed using those cells presumed to be the principal site of HCG action. Other possible experiments are discussed in the next chapter - General Discussion.

CHAPTER 8

INFLUENCE OF 5α-REDUCED ANDROGENS ON OESTRADIOL AND PROGESTERONE SECRETION BY GRANULOSA CELLS FROM PREPUBERTAL RATS PRIMED WITH PREGNANT MARES' SERUM GONADOTROPHIN

8.1 INTRODUCTION

In 1970, Eckstein, Mechoulam and Burstein identified 5α-androstane-3α, 17β-diol as a principal metabolite when ^{3}H-pregnenolone was incubated with ovarian homogenates from immature female rats. They observed no formation of the androstanediol when ovarian homogenates prepared from mature rats were used under a similar condition. It is well established that 5α-reduced androgens, notably 5α-dihydrotestosterone (DHT) will enhance 'progestin' secretion from isolated granulosa cells (Armstrong and Dorrington, 1976; Lucky, Schreiber, Hillier, Schulman and Ross, 1977). More recently, Nimrod, Rosenfield and Otto (1980) reported on the relative potencies of 5α-reduced androgens in augementing progestin secretion from granulosa cells isolated from immature hypophysectomized oestrogen-primed rats, while Hillier et al. (1980a) have reported that 5α-reduced androgens inhibited the aromatase activity in the granulosa cells isolated from preovulatory follicles of adult rats.

The present studies were carried out to examine the effect of the 5α-reduced androgens which have been identified in the ovary in immature rats on the aromatase activity in the granulosa cells of immature rats, over a short time period. The effects on the aromatization of androgen have been compared with those on progesterone secretion by the same cells.

8.2 METHODS

26-day old rats were injected subcutaneously with 5 iu PMSG at 10.00 h. The animals were killed between 10.00 and 12.00 h on day 28 (before the endogenous LH surge). Granulosa cells were prepared as described previously (2.11).

Incubation Procedure

100 ul aliquots of suspended granulosa cells were transferred into a series of 11.5 x 36 mm glass vials containing an additional 900 µl KRB to which steroid or protein hormones had previously been added. Incubations were carried out in quadruplets for 4 h at 37°C under an atmosphere of 95% O_2:5% CO_2 in a shaking water bath set at 120 oscillations/min. At the end of the incubation, the cells were sedimented by centrifugation and the medium stored at -20°C. Subsequently oestradiol and progesterone were measured by specific radioimmunoassays as described in section 3.4.

8.3 RESULTS

8.3.1 Effects of Δ^4- androgens and Δ^4-5α reduced androgens on oestradiol secretion from granulosa cells

The amount of oestradiol secreted by granulosa cells in the absence of added substrate was low (0.85 ± 0.10 pmoles/10^5 cells/4h). This secretion was significantly raised ($p < 0.001$) by the addition of either 1 x 10^{-7}M testosterone (to 10.6 ± 0.9 pmoles/10^5 cells/4h) or androstenedione (to 5.3 ± 0.1 pmoles/10^5 cells/4h). The greatest stimulation of oestradiol secretion by testosterone or androstenedione was observed when the androgens were present at 1 x 10^{-6}M. In contrast, the addition of

the four Δ^4-5α-reduced androgens did not stimilate oestradiol accumulation in the medium containing the granulosa cells. These data are compared in figure 8.1.

Additions of 1 x 10^{-7}M testosterone and 1 x 10^{-6}M Δ^4-5α-reduced androgens were used in subsequent experiments.

8.3.2 Effect of 5α-reduced androgens on oestradiol and progesterone secretion from granulosa cells in the presence of testosterone

The four 5α-reduced androgens examined all caused a potent inhibition of the oestradiol secreted by granulosa cells in the presence of exogenous testosterone (1 x 10^{-7}M) as aromatizable substrate. 5α-androstanedione, at a concentration of 1 x 10^{-6}M, caused the most marked inhibition, by 76.6% of the level achieved with testosterone in the medium ($p < 0.001$). Likewise, 5α-dihydrotestosterone resulted in a significant inhibition (by 71.9%, $p < 0.001$), as did 5α-androstane-3α-17β diol (25.7%, $p < 0.02$) and 5α-androstane-3β-17β, diol (41.6%, $p < 0.01$). The results are shown in Figure 8.2.

In contrast to this effect on oestradiol secretion from granulosa cells, the 5α-reduced androgens generally potentiated the secretion of progesterone from granulosa cells. This response was not modified by adding exogenous testosterone. Androstanedione (in the absence of exogenous testosterone) increased the amount of progesterone which accumulated in the medium from 5.75 $\pm$ 0.62 to 20.75 $\pm$ 2.03 pmoles/10^5 cells/4 h ($p < 0.01$). This represents an increase of 261% from the secretion in the absence of testosterone. With both androstanedione and testosterone added to the medium, the progesterone accumulating after 4 h reached 20.0 $\pm$ 1.9 pmoles/10^5 cells; which was significantly

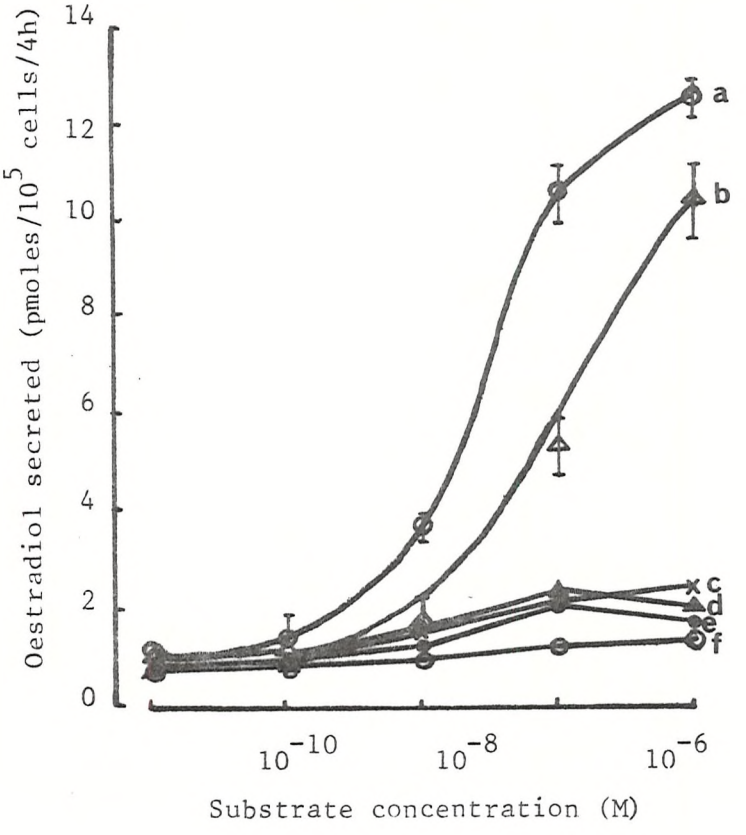
greater than the amount accumulating with the addition of testosterone alone. The levels of progesterone attained in the medium were still significantly greater ($p < 0.01$; representing 247.8%) than the levels attained without addition of any androgens.

The other 5α-reduced androgens followed a similar pattern of responses with respect to progesterone secretion. In all instances, the addition of the 5α-reduced androgen caused a marked stimulation of progesterone accumulating in the medium, compared with the level observed in the absence of added androgen ($p < 0.01$ for 5α-dihydrotesterone (231.3%); $p < 0.01$ for 5α-androstane-3α-17β, diol (147.8%); $p < 0.01$ for 5α-androstane-3β, 17β-diol (255.5%). The stimulation of progesterone accumulation was still observed in the presence of testosterone. These data are summarized in Figure 8.2 .

8.3.3 Effects of 5α-reduced androgens on oestradiol and progesterone secretion with the addition of oFSH and testosterone

The effect of the 5α-reduced androgens on the pattern of oestradiol and progesterone which accumulated in the incubation medium over 4 h was not substantially affected by adding oFSH to the incubates. Thus the oestradiol which accumulated with the addition of both testosterone (1×10^{-7}M) and oFSH (0.1 μg/ml) was 24.97 ± 1.82 pmoles/10^5 cells/4 h. This was reduced to 13.04 ± 0.75 pmoles/10^5 cells/4 h by adding 1×10^{-6}M 5α-dihydrotestosterone ($p < 0.001$; 47.8%). The other 5α-reduced androgens followed a similar pattern of responses with respect to oestradiol secretion. In all instances, addition of the

FIGURE 8.1 OESTRADIOL SECRETED BY GRANULOSA CELLS ISOLATED FROM PMSG-PRIMED IMMATURE RATS IN THE PRESENCE OF (a) TESTOSTERONE, (b) ANDROSTENEDIONE, (c) 5α-DIHYDROTESTOSTERONE, (d) 5α-ANDROSTANEDIONE, (e) 5α-ANDROSTANE-3α,17β-DIOL, OR (f) 5α-ANDROSTANE-3β,17β-DIOL



Granulosa cells were collected from the large antral follicles of immature rats primed with PMSG, as described in the text, (p55). Androgens were added to the incubation medium (Kreb's Ringer bicarbonate buffer, pH 7.4) at concentrations of 1×10^{-10} to 1×10^{-6} M Oestradiol accumulating in the medium during a 4 h incubation period was measured by a specific radioimmunoassay, described in the text. Values are expressed as means ± SEM of four incubates.

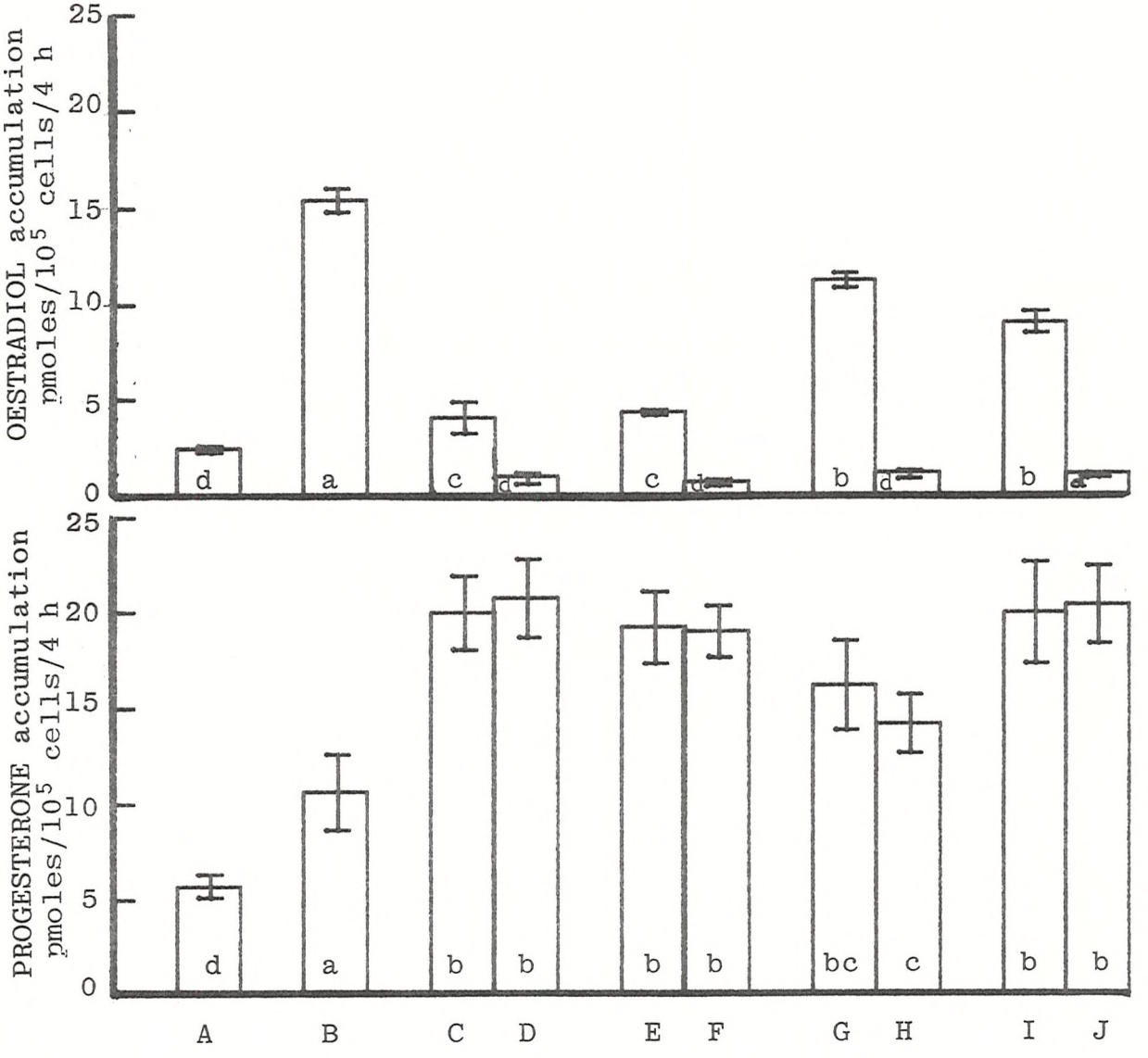
Legend Fig. 8.2

The effect of 5α-reduced androgens on the ability of the granulosa cells to secrete OESTRADIOL and PROGESTERONE in the presence of exogenous testosterone, is shown.

Steroid secreted:

(A) without exogenous testosterone; (B) in the presence of 1×10^{-7} M testosterone; (C) in the presence of both 5α-androstanedione (1×10^{-6} M) and testosterone or (D) in the presence of 5α-androstanedione alone; (E) in the presence of both 5α-dihydrotestosterone (1×10^{-6} M) and testosterone or (F) 5α-dihydrotestosterone alone; (G) in the presence of both 5α-androstane-3α, 17β-diol and testosterone or (H) 5α-androstane-3α, 17β-diol alone; (I) in the presence of 5α-androstane-3β, 17β-diol and testosterone or (J) of 5α-androstane-3β, 17β-diol alone.

FIGURE 8.2 OESTRADIOL AND PROGESTERONE ACCUMULATING IN THE MEDIUM CONTAINING GRANULOSA CELLS FROM PMSG-PRIMED IMMATURE RATS: THE EFFECT OF 5α-REDUCED ANDROGENS IN THE PRESENCE OR ABSENCE OF TESTOSTERONE.



The granulosa cells were prepared from large antral follicles of PMSG-primed immature rats as described in the text. Testosterone (1×10^{-7}M) or 5α-reduced androgens (1×10^{-6}M) were added at the beginning of the incubation period. Oestradiol and progesterone accumulating in the incubation medium during 4 h incubation were measured by specific radioimmunoassays as described in the text. Results are expressed as means ± SEM values on duplicate analyses of four incubates.

The data were examined by analysis of variance and the means compared by the Duncan's multiple Range test.

Mean values without a common Roman letter within the column are significantly different ($p < 0.05$).

Legend Fig. 8.3

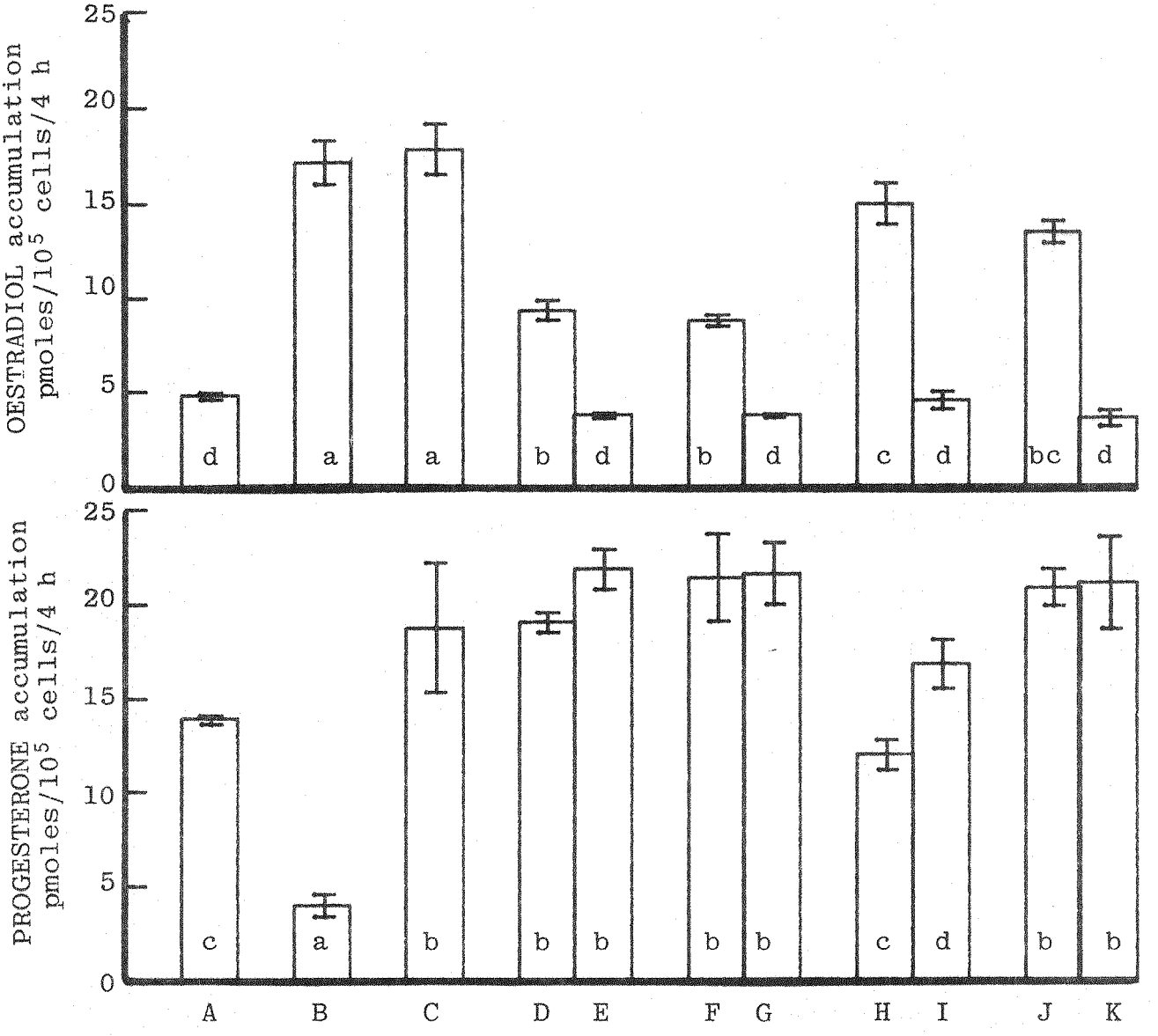
The effect of 5α-reduced androgens (1×10^{-6} M) and ovine-FSH (0.1 μg/ml) on the ability of the granulosa cells to secrete OESTRADIOL or PROGESTERONE in the presence or absence of exogenous testosterone (1×10^{-7} M), is shown.

Steroids secreted:

(A) with the addition of oFSH; (B) with the addition of exogenous testosterone; (C) with the addition of oFSH and testosterone; (D) in the presence of dihydrotesterone with testosterone and oFSH or (E) in the presence of dihydro-testosterone and oFSH; (F) in the presence of 5α-andro-stanedione with testosterone and oFSH or (G) in the presence of 5α-androstanedione and oFSH; (H) in the presence of 5α-androstane-3α, 17β-diol with testosterone and oFSH or (I) in the presence of 5α-androstane-3α, 17β-diol and oFSH; (J) in the presence of 5α-androstane-3β, 17β-diol with testosterone and oFSH or (K) in the presence of 5α-androstane-3β, 17β-diol and oFSH.

The amount of oestradiol and progesterone secreted without any added testosterone, 5α-reduced androgen or oFSH was the same as that in the previous experiment (Figure 8.2). The significance of the differences of the mean values for these data, in the analysis of variance compared by the Ducan's multiple range test, using the nomenclature in this figure were, for oestradiol d, and for progesterone, a.

FIGURE 8.3 OESTRADIOL AND PROGESTERONE ACCUMULATING IN THE MEDIUM CONTAINING GRANULOSA CELLS FROM PMSG-PRIMED IMMATURE RATS: THE EFFECT OF 5α-REDUCED ANDROGENS AND oFSH IN THE PRESENCE OR ABSENCE OF EXOGENOUS TESTOSTERONE.



The granulosa cells were prepared as described under Fig. 8.2. Testosterone (1×10^{-7}M), oFSH (0.1 µg/ml) or the 5α-reduced androgens (1×10^{-6}M) were added at the beginning of the incubation period. Oestradiol and progesterone which accumulated in the incubation medium during 4 h incubation were measured by specific radioimmunoassays as described in section 3.4. Results are expressed as means ± SEM on duplicate analyses of four incubates.

The data were examined by analysis of variance and the means compared by the Duncan's multiple Range test.

Mean values without a common Roman letter within the column are significantly different ($p < 0.05$).

5α-reduced androgen caused a significant inhibition of oestradiol accumulating in the medium. The percentage inhibition of these steroids were 51.0 (5α-androstanedione), 16.4 (5α-androstane, 3α-17β-diol) and 24.8 (5α-androstane-3β-17β-diol) respectively (incubations in the presence of testosterone and oFSH = 100%). In the absence of exogenous testosterone, oFSH did not stimulate oestradiol accumulation, and no effect of the 5α-reduced androgens was observed on the basal level of oestradiol secreted in the absence of oFSH.

The amount of progesterone accumulating in the presence of oFSH and testosterone was high (26.28 ± 4.76 pmoles/10^5cells/4 h). The presence of oFSH in the medium did not significantly affect the stimulation of progesterone secretion caused by any of the four 5α-reduced androgens in either the presence or absence of testosterone. These data are summarized in Figure 8.3 .

8.4 DISCUSSION

The present results clearly show that 5α-reduced androgens will inhibit the conversion of exogenous testosterone to oestradiol by granulosa cells isolated from PMSG-primed immature rats' ovaries, and furthermore that they will stimulate the accumulation of progesterone in the medium containing the same cells. The effects of the 5α-reduced androgens which have been shown in the present studies, are generally similar to those of other comparable studies (Lucky et al., 1977; Hillier et al., 1980a). However, Nimrod et al., (1980) found that testosterone (0.5 μg/ml) significantly increased progestin production, while the 5α-reduced androgens, 5α-dihydrotestosterone, and the 5α-androstanediols were not so potent. This is in marked contrast to the present studies where testosterone (1 x 10^{-7}M) had no significant

effect on progesterone secreted by the granulosa cells although the 5α-reduced androgens (5α-dihydrotestosterone, 5α-androstanedione and 5α-androstane-3β-17β diol) did. A possible explanation for this discrepancy may be because Nimrod et al., (1980) used granulosa cells from hypophysectomized immature rats maintained for 20 h in culture with exogenous oFSH, compared with our preparation.

8.4.1 Comparison of the relative effects of testosterone and the 5α-reduced androgens on the secretion of oestradiol and progesterone by isolated granulosa cells

The preceding results have illustrated that testosterone and the 5α-reduced androgens have differential effects on the secretion of oestradiol and progesterone by isolated granulosa cells. These differences are now summarized.

Treatment	Steroids secreted pmole/10^5 cells/4 h	
	Oestradiol	Progesterone
1. Granulosa cells + testosterone (T, 1 x 10^{-7}M)	15.42 ± 0.61	10.05 ± 2.02
Granulosa cells alone (control)	2.42 ± 0.24	5.8 ± 0.62
2. Granulosa cells + T + 1 x 10^{-6}M 5α-androstanedione (5α-A)	4.04 ± 0.82	20.04 ± 1.92
Granulosa cells + 5α-A	0.98 ± 0.24	20.75 ± 2.03
3. Granulosa cells + T + 1 x 10^{-6}M 5α-dihydrotestosterone (5α-DHT)	4.32 ± 0.16	19.25 ± 1.84
Granulosa cells + 5α-DHT)	0.64 ± 0.16	19.05 ± 1.32
4. Granulosa cells + T + 1 x 10^{-6}M 5α-androstane, 3α-17β, diol (αβ)	11.22 ± 0.42	16.25 ± 2.32
Granulosa cells + αβ	1.08 ± 0.20	14.52 ± 1.54
5. Granulosa cells + T + 1 x 10^{-6}M 5α androstane, 3β-17β-diol (ββ)	9.04 ± 0.56	20.04 ± 2.64
Granulosa cells + ββ	1.04 ± 0.12	20.44 ± 2.02

This data shows that testosterone (1×10^{-7}M) stimulated oestradiol accumulation in the medium approximately 6 fold, while testosterone had little effect on progesterone accumulation (stimulating it 2-fold). 1×10^{-6}M 5α-androstanedione (in the presence of 1×10^{-7}M testosterone), reduced oestradiol levels to x 2 greater than basal levels, while stimulating progesterone accumulation x 2 above that in the presence of testosterone alone. This same 5α-reduced androgen also stimulated progesterone accumulation x 4 above the levels achieved by the isolated granulosa cells alone. The effects of 5α-dihydrotestosterone were very similar to those of 5α-androstanedione, but 5α-androstane, 3α-17β,diol was less potent than the two preceding androgens, whereas its 3β epimer was as effective as 5α-dihydrotestosterone in stimulating progesterone accumulation, but not as effective in reducing either testosterone stimulated or endogenous oestradiol secretion. This last point is important since it may be interpreted to mean that the 5α-reduced androgens can have differential effects on the aromatizing enzymes (possibly directly), on the supply of aromatizable substrate (possibly by inhibiting the C-17-20 lysase) or on the synthesis/secretion of progesterone from cellular precursors (possibly cholesterol side-chain cleavage enzymes or mitochondrial uptake/metabolism of cholesterol).

Another aspect of the differential effect of the 5α-reduced androgens is that they may be metabolized at different rates within the ovarian tissues. This has been suggested by the work of Nimrod, et al., (1980). These authors found that the 5α-androstane-3α,17β diol was broken down more rapidly than 5α-dihydrotestosterone. This could explain the apparently lesser effect of the androstanediol in these studies. The concen-

trations of the added exogenous 5α-reduced androgens at the end of the experiment, in the tissue and medium, was not measured, although this could have been done by adding trace amounts of each of the radioactive 5α-reduced androgens to the incubates at the beginning of the experiment. At the end of the incubation, the radioactive isotope could have been purified (by TLC and subsequently recrystallized to constant specific activity with authentic steroid). Nevertheless, a difference in the rate of metabolism does not really explain the differential effects of the 5α-reduced androgens on oestradiol and progesterone secretion (unless of course, the metabolites of the 5α-reduced androgens are also active). In this context, it is known that the 5α-reduced androstanediols are present in the circulation principally as their sulphates (Ravid and Eckstein, 1976), but there are no reports at present either on the sites where the sulphation occurs (though presumably this is in the liver and/or kidney), nor on the effects of the conjugates on ovarian steroidogenesis.

8.4.2 Physiological implications of the effects of 5α-reduced androgens on oestrogen and progesterone secretion

I have argued previously that granulosa cells isolated from the pre-ovulatory follicles of PMSG-primed rats probably behave, functionally, in a similar way to those in pre-ovulatory follicles from adult rats at pro-oestrus (see Chapter 5). The effects of 5α-reduced androgens observed in these experiments are very similar to those observed in the experiment of Hillier et al., (1980a), in which granulosa cells prepared from adult rats were used.

However, the effect of 5α-reduced androgens in immature rats are particularly significant since these androgens have been identified as major metabolites of pregnenolone in the ovaries of prepubertal rats (Eckstein, et al., 1970). Furthermore, the 5α-reduced androgens have been implicated in controlling the onset of puberty, although generally it has been suggested that this is due to their action on the hypothalamus (Eckstein, Shani, Ravid and Goldhaber, 1981).

The levels of both testosterone (1×10^{-7}M) and 5α-reduced androgens (1×10^{-6}M) which have been used in these experiments are thought to be within the range found in follicular fluid (McNatty, Makris, De Grazia, Osathanondh and Ryan, 1979). This of course implies that the 5α-reduced androgens are in contact with granulosa cells, under normal circumstances, at levels which here have been found to have significant effects on the steroid metabolism. Furthermore, it implies that the regulation of 5α-reductase in the micro-environment of the follicle, may represent an important control step in the regulation of follicular oestrogen synthesis, and ultimately therefore, also in the regulation of the sensitivity to gonadotrophins. Very little is known about the 5α-reductase activity within a single follicle, although within the whole ovary of immature rats, there is both direct and indirect evidence that it may be controlled by gonadotrophins. Directly, Eckstein and Ravid (1979) showed that the endogenous LH surge causing the first (pubertal) ovulation, was also associated with a rapid decrease in the synthesis of androstanediols. Indirectly, Suzuki et al. (1978) showed that the levels of 5α-reductase decreased in immature rats treated with PMSG. This evidence is indirect since, as already discussed, PMSG stimulates many aspects of follicular

development and steroidogenesis (see Chapter 5). But in both these experiments, whole ovaries have been used, and since under both circumstances (natural or induced puberty), the size of follicles and the proportion of cells within the ovaries is changing, the 5α-reductase may represent the activity of only a few ovarian/follicular cells at any one time. Unlike steroid dehydrogenase activity, there is at this time no means of directly visualizing 5α-reductase activity in ovarian tissues. It might be possible to measure 5α-reductase directly in isolated granulosa cells, thecal cells, or ovarian follicles, using isotope-conversion techniques as suggested for other purposes in this thesis. However, the subsequent separation of the 5α-reductase metabolites is not simple, since the isomers have rather similar chromatographic mobilities.

GENERAL DISCUSSION

Many of the biochemical events which precede ovulation have been studied, defined and documented. In particular, the changing profile of steroid secretion induced by the pre-ovulatory LH surge, has attracted considerable interest (Tsafriri et al., 1973; Lindner et al., 1974; Hillensjo et al., 1976; Ahren et al., 1979). Since most of these reports have already been discussed, to avoid repetition, I shall raise here only those salient points which need to be discussed in juxtaposition.

Treating immature female rats with PMSG provides a well controlled means for synchronizing the transition of small antral follicles with a few layers of granulosa and thecal cells to more advanced levels of follicular maturity associated with the development of numerous pre-ovulatory Graafian follicles and the subsequent formation of corpora lutea (Fig. 5.3D). In conjunction with our in vitro procedures for examining the steroidogenic capacity of the intact ovary or of granulosa cells we have correlated the histological development of the ovary with changes in the circulating levels of reproductive steroids.

Our finding that PMSG promoted the growth of the follicles is consistent with the FSH-like property of this gonadotrophin (Cole, 1936; Schulster et al., 1976). We observed that the time course of the development of pre-ovulatory Graafian follicles (as previously mentioned) induced by PMSG was similar to that which has been reported to follow the injections of FSH into oestrogen-primed hypophysectomized rats (Erickson and Hseuh, 1978). In the same experiment these authors observed that 48 h after treating the rats with FSH (when the morphology of the follicles

was similar to the pre-ovulatory follicles in adult rats on the morning of pro-oestrus), granulosa cell aromatase activity reached a maximal value, and this value was comparable to that found in granulosa cells of mature Graafian follicles isolated from adult rats at pro-oestrus. From these observations it would seem that the induction or activation of granulosa cell aromatase activity is a function of FSH acting during the course of normal pre-ovulatory follicular development (Hillier et al., 1980b).

Studies with granulosa cells isolated from PMSG-primed immature rats showed that the amount of oestradiol secreted by these cells was low in the absence of aromatizable androgen (testosterone) in the incubation medium. Neither FSH nor LH stimulated oestradiol secretion from these cells in the absence of testosterone. This result is consistent with the findings of Dorrington, Moon and Armstrong (1975) and Fortune and Armstrong (1978). The failure of isolated granulosa cells to synthesize oestradiol may be due to their inability to synthesize aromatizable androgens, since androgens are produced predominantly by the LH-stimulated thecal tissue (Fortune and Armstrong, 1977). This conclusion was supported by the finding that isolated granulosa cells synthesized substantial amounts of oestradiol in the presence of exogenous testosterone (1×10^{-7}M) with or without FSH (Fig. 4.1B). Again this result is in accord with the data of Fortune and Armstrong (1978) and Hillier et al., (1980a) who found that granulosa cells from immature rats primed with PMSG, or granulosa cells isolated from adult rats at pro-oestrus, produced oestradiol in the presence of testosterone. However, our results and those of Hillier et al., (1980a) are in contrast

with the results of Dorrington et al., (1975) who found that granulosa cells from hypophysectomized immature rats secrete oestradiol only in the presence of both FSH and testosterone. An explanation for this difference may be that whereas Hillier et al., (1980a) used intact pro-oestrus rats (in which the endogenous gonadotrophins, LH and FSH, would have been high), Dorrington et al., (1975) used hypophysectomized oestrogen-treated immature rats (with no endogenous gonadotrophin). By using the model of the PMSG-primed rat, it is very likely that we had induced both FSH and LH receptors in the granulosa cells, during the 48 h following the injection of PMSG. It has been reported that FSH will induce its own receptors in granulosa cells especially in the presence of oestradiol (Richards, Ireland, Rao and Reichert, 1976). We know that the PMSG used here as previously mentioned, contained FSH-like activity because of the ability of the hormone to stimulate apparently normal follicular development (Fig. 5.3). This result is in accordance with the earlier studies of Lostroh and Johnson (1966) and Armstrong and Papkoff (1976) who demonstrated that only if FSH and LH were administered simultaneously did all stages of normal follicular development occur, in conjunction with oestrogen formation, in hypophysectomized immature rats. This FSH-like property of PMSG was further confirmed by its ability to stimulate oestradiol secretion by granulosa cells in the presence of testosterone (Table 4.1.B). Again, uterine weights in the PMSG-primed rats increased over the 48 h from 70.7 $\pm$ 9.0 mg to 130.0 $\pm$ 13.8 mg ($P < 0.02$) indicating an increased level of oestrogen secretion, while plasma oestradiol levels increased from 1.02 $\pm$ 0.09 x 10^{-13} moles/ml to 4.1 $\pm$ 0.3 x 10^{-13} moles/ml

($P < 0.01$) over the same period (Table 5.2.1 and Fig. 5.1). Androgen secretion also increased over this period: plasma testosterone levels rose from 4.62 $\pm$ 0.43 pmoles/ml on day 25, before the PMSG injection, to 10.22 $\pm$ 1.93 pmoles on day 27 in the PMSG treated rats, compared with 6.25 $\pm$ 0.91 pmoles/ml on day 27 in saline injected controls ($P < 0.02$, Fig. 5.1C). This is relevant since androgen has been implicated in the control of follicular maturation (Goff, Leung and Armstrong, 1979; Hillier et al., 1980a).

The work, reported in Table 5.3, shows that ovaries from immature rats primed with PMSG for 48 h secrete predominantly oestradiol and testosterone during a 4 h incubation period. Histological studies (Fig. 5.3C) revealed that these ovaries contained numerous large pre-ovulatory Graafian follicles. These are the follicles considered to be responsible for the large quantities of oestrogens secreted by the ovary into the peripheral circulation during oestrus (Baird and Fraser, 1975; Makris and Ryan, 1975). These ovaries can be compared with the Graafian follicles of adult pro-oestrus rats extirpated before the endogenous LH surge and cultured for 4-12 h. which also secrete principally oestradiol and androstenedione (Tsafriri et al., 1973; Hillensjo et al., 1976). It has also been shown that the addition of HCG to incubates of ovaries from immature rats primed with PMSG for 48 h, caused a marked increase in the secretion of oestradiol and testosterone, similar to that found for cultured Graafian follicles of adult pro-oestrus rats (Tsafriri et al., 1973 and Lindner et al., 1974). Similarly, the ovaries from 72 h PMSG-primed animals and the isolated Graafian follicles harvested after the expected LH surge, secreted predominantly progesterone without any increase in testosterone or

oestradiol secretion, when HCG was added to the medium. However, when the endogenous LH surge was inhibited (by sodium pentobarbitone injected at 14.00 h on the second day after the PMSG injection), the ovaries from the 72 h PMSG-primed animals secreted predominantly oestradiol and testosterone and only small amounts of progesterone. These rats (in which the LH surge had been inhibited) had no ova in their oviducts. Thus the profile of steroids secreted at this time resembles that in the 48 h primed rats (Fig. 5.2). This lends support to the hypothesis that the LH/FSH surge and the events accompanying it, are responsible for inducing the inhibition of oestrogen synthesis which occur around the time of ovulation.

In spite of the differences in experimental models between our ovaries from 48 h PMSG-primed animals and the Graafian follicles of adult pro-oestrus rats which were used by Lindner et al., (1974) (e.g. duration of incubation, incubation medium composition, oxygen tension), a similar profile of steroids secreted was obtained. These results suggest that the two types of pre-ovulatory follicles: those harvested on the morning of pro-oestrus of adult cycling rats, and ovaries taken from immature rats primed with PMSG for 48 h, are very similar both in their secretion of steroids in vitro and the responses to LH or HCG. These results are also consistent with the earlier report (Hillensjo et al., 1976), on the steroidogenesis in isolated peri-ovulatory follicles of PMSG-primed immature rats, showing that exposure to the preovulatory gonadotrophin surge resulted in the inhibition of androgen and oestrogen secretion and stimulation of progesterone biosynthesis.

It is not fully understood which mechanism(s) regulate follicular progesterone production before the endogenous LH surge, nor precisely how LH promotes the 'transition' to increased progesterone production. Equally unclear is which factors are involved in reducing the androgen and oestrogen secretion during the time interval between the LH surge and ovulation, although some possibilities will be raised in the present discussion.

It is known that granulosa cells which have luteinized in culture, and corpora lutea, secrete progesterone. Furthermore, it is known that the LH stimulated progesterone production by luteal cells is mediated through c'AMP (Channing and Tsafriri, 1977). As previously stated, it is uncertain at which level(s) LH acts to inhibit the formation of androgen and oestrogen (Leung and Armstrong, 1980). It has been suggested that LH may stimulate the production of a protein which inhibits the enzyme involved in the cleavage of the C17-side chain of progesterone (17α-hydroxylase and/or 17:20 lysase), thereby resulting in a simultaneous sharp decline of both androgen and oestrogen production (Lieberman et al., 1975). In addition, Katz and Armstrong (1976) have suggested that LH may inhibit oestrogen production by reducing ovarian androgen aromatase activity. However, the drastic decline in the amount of oestradiol secreted by ovaries of animals which had been treated with PMSG for 72 h earlier was not accompanied by a parallel sharp decline in the amount of testoterone secreted by the whole ovary in vitro, nor in the peripheral plasma testosterone levels in vivo (Table 5.3 and Fig. 5.1). This is consistent with the suggestion that the lack of aromatizable substrate may not be the only cause of

decreased secretion of oestradiol which was observed.

We have earlier shown that the ovaries from rats primed with PMSG for 48 h secrete high levels of oestradiol. The addition of testosterone to incubation medium containing these ovaries resulted in a marked increase in this oestradiol secretion (Fig. 6.2). We know that these ovaries contained a mixed population of follicles: large pre-ovulatory follicles, medium sized follicles and some atretic follicles (see Fig. 5.3.C). It is also known that atretic follicles secrete high levels of aromatizable androgens (Moor, 1977; Moor, Hay, Dott and Cran, 1978). In view of the above observations, it seems likely that atretic antral follicles (together with Stromal and/or interstitial tissue) may be an important supplementary source of androgen within the ovary. However, there is also evidence that androgen production occurs locally at a rate which is closely 'tuned' to that of its aromatization by the granulosa cells within the same follicle (Hillier, 1981).

Furthermore, exogenous androgen has been implicated in the induction of atresia of preantral follicles (although its role in this process in antral follicles has not been substantially reported) (Hillier and Ross, 1979). However, Louvet, Harman and Ross (1975) postulated that ovarian androgen, secreted in response to LH, restrained the mitogenic action of oestrogen on the granulosa cells of preantral follicles. Thus, the ultimate fate of follicles may well depend on the balance between androgen synthesis and oestrogen formation at a critical stage of development; an early induction of aromatase in granulosa cell

by FSH (Dorrington et al., 1975) would reduce the chances of androgen-induced atresia while favouring oestrogen-induced follicular growth and induction of LH receptors (See Chapter 1). (Lindner, Amsterdam, Salomon, Tsafriri, Nimrod, Lamprecht, Zor and Koch, 1977).

By using the 'whole ovary' model, as presented here, we cannot clearly localize the cellular site(s) of action of the LH-induced decline in oestrogen secretion. However, studies with isolated thecal and granulosa cells in tissue culture indicate that the thecal tissue is the major source or follicular androgen while the granulosa cells are the major source of follicular oestrogen. Also, as stated previously, the granulosa cells aromatize the androgens produced by the LH-stimulated thecal tissue (Fortune and Armstrong, 1977, 1978). Thus, although LH presumably exerts an inhibitory effect on the thecal cells to 'shut down' androgen production, LH may have a dual effect on the granulosa cells: LH may stimulate the production of progesterone by granulosa cells while at the same time inhibiting their capacity to aromatize androgen (Goff and Henderson, 1979).

It has been suggested that the principal action of HCG on the pre-ovulatory ovaries may be to raise the endogenous levels of oestradiol precursor (testosterone) and it is this which in turn increases the amount of oestradiol formed and accumulating in the medium. This hypothesis was supported by the observation that in the presence of increasing concentrations of exogenous testosterone increased amounts of oestradiol accumulated in the medium (Table 6.1). However, the ovaries of saline treated animals produced negligible amounts of oestradiol during a 4 h

incubation in the presence of exogenous testosterone. A possible explanation of this is a lack of activated aromatase activity in the ovaries from saline treated animals. It is known that following a single injection of 10 iu PMSG to immature rats, the activity of the androgen 5α-reductase decreases significantly over the ensuing 48 h, while the activities of the C17-C20 lysase and 17α-hydroxylase enzymes are enhanced (Suzuki et al., 1978). There could therefore be a combined influence of a reduction in the levels of Δ^4-5α-reductase, together with increased levels of enzymes involved in testosterone synthesis, which might act synergistically to further enhance the aromatization of (both exogenous and endogenous) testosterone observed in the PMSG primed ovaries, as suggested by the scheme shown in Fig. 9.1.

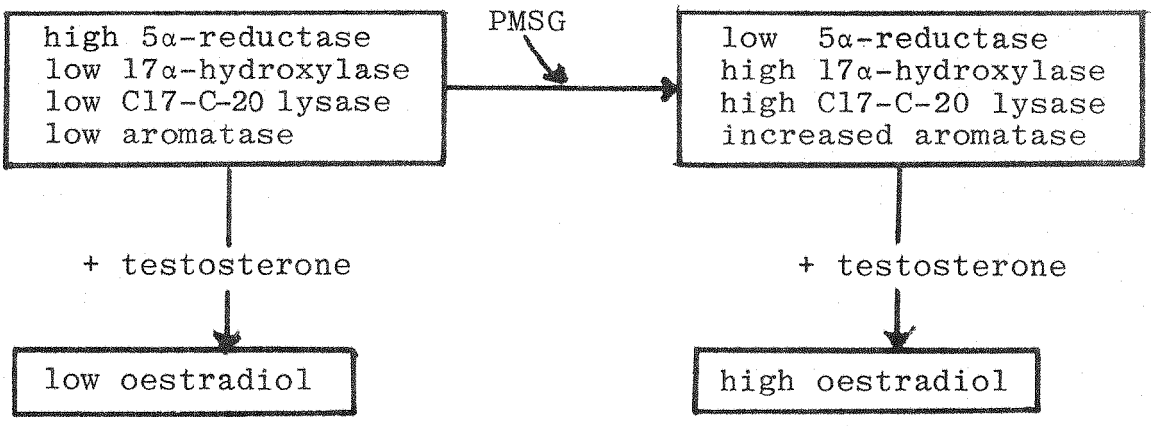


FIGURE 9.1 Schematic representation of the effect of PMSG treatment on the activities of: Δ^4-5α reductase; 17α-hydroxylase; C-17,C-20 lysase; and androgen aromatase enzymes. These data are taken from Suzuki et al., 1978. In their experiments the 10,000 g supernatant fluid prepared from 23 day old rats treated with PMSG (10 iu) 48 h previously was used. The incubation was carried out in the presence of appropriate labelled substrates and the metabolites formed were initially identified by chromatographic mobility and subsequently by recrystalization to a constant specific activity with authentic steroid. The relative activities of the different enzymes is based on the conversion of these labelled precursors. The scheme which these authors preposed is comparable with our present data.

Some interesting possibilities for the effects of 5α-reduced androgens on ovarian functions in the prepubertal rat can be envisaged. Eckstein, Mechoulam and Burstein (1970) reported that 5α-androstane-3α,17β-diol (3α-A) accumulated as a major metabolite when ^{3}H-pregnonolone was incubated with ovarian homogenates from immature rats. They observed no formation of this androgen, however, when an ovarian preparation obtained from pubertal rats (40 days of age) was used under the same conditions (Eckstein et al., 1970). The 3α-A , as well as its 3β epimer (3β-A), are known to be present in peripheral blood of immature female rats in concentrations of 100 ng/ml (present mainly as sulphate conjugates, Ravid and Eckstein, 1976), but are undetectable in adult rats (Eckstein and Ravid, 1974). These androgens are also known to delay the onset of puberty in immature rats (Eckstein, 1975), it is generally assumed by their action on the hypothalamus (Eckstein, Ravid and Goldhaber, 1981). However, our recent studies (Ebong and Peddie, 1980) suggest that part of their role in regulating puberty may be due to a direct effect on the ovary, by inhibiting oestradiol secretion from maturing antral follicles.

Furthermore, it has been demonstrated that Δ^{4}-5α-reductase activity in ovarian homogenates from immature rats is stimulated by LH, but not by FSH, oestradiol or androgens (Terakawa, Kondo, Aono, Kurachi and Matsumoto, 1978). It can be envisaged that the occasional surges of LH seen in immature rat from day 21 (MacKinnon, Puig-Duran and Laynes, 1978) may sustain the levels of this enzyme <u>in vivo</u>, thus ensuring that the 5α-reduced androgens present in the ovary help to suppress oestradiol formation until the time of puberty.

It has been widely reported that protein synthesis may play a role in LH/HCG stimulated steroidogenesis (Lindner et al., 1974; Lieberman et al., 1975 and Younglai, 1975). The addition of cycloheximide to ovarian incubates suppressed the HCG-induced testosterone and oestradiol secretion (Fig. 6.1) which is consistent with such a role. Implicit in the role of protein synthesis is the assumption that the action of the inhibitors was solely due to their effect on protein synthesis, and not due to a non specific 'toxic' effect. Some measure of support for the specific action of cycloheximide comes from the observation that three different inhibitors of protein synthesis were equally effective in blocking the actions of LH, while an amino-nucleoside analogue of puromycin was not effective (Lindner et al., 1974). Furthermore, studies by Davis and Garen (1968) with adrenal cortical cells had shown that cycloheximide inhibits the enzyme(s) responsible for the conversion of cholesterol to pregnenolone. This conclusion could explain the results shown in Fig. 7.1 (differential inhibition of ovarian steroids by cycloheximide); when inhibition of protein synthesis occurs only a small amount of pregnenolone may be available as substrate for progesterone synthesis, so that progesterone production drastically falls. Testosterone and oestradiol secretion may depend upon a nascent pool of precursors, the concentration(s) of which may not be affected by the low concentration of cycloheximide (5 µg/ml).

It is apparent from these studies that protein synthesis and HCG-induced steroidogenesis are both suppressed by cycloheximide, implicating the involvement of a labile protein in mediating the HCG-induced response. This protein must however

be involved at a stage prior to the aromatization stage, since the production of oestradiol by ovaries in the presence of testosterone was not significantly affected by cycloheximide, (Fig. 6.2). However, the precise role of the protein remains to be elucidated. Further possible courses of investigation would be to examine the effects of inhibitors of protein synthesis and of RNA synthesis on the ability of different ovarian cell types to respond to exogenous hormone stimulation. This could be carried out by using experimental procedure similar to the one described in Chapter 7.

Furthermore, it sould be established whether de novo RNA synthesis or the translational stage of protein synthesis was stimulated by gonadotrophin and whether the proteins formed were effective on the activity of cholesterol esterase, on the mitochondrial uptake of cholesterol, or on the side chain cleavage enzymes located in the mitochondria and hence the formation of pregnenolone. This could be done by preparing cytosol or mitochondrial suspensions from ovarian cells by differential centrifugation, and incubating them with tritiated precursors. The tritiated metabolite(s) e.g. pregnenolone, could then be isolated initially by thin layer chromatography and subsequently identified by recrystalization to a constant specific activity with authentic steroid.

Since our results indicate that protein synthesis may be involved in the HCG stimulation of steroidogenesis, it would be interesting to investigate whether there is any evidence for the formation of phosphorylated proteins or of dephosphorylation during the period of stimulation. This could be done by incubating the ovarian tissues with ^{32}P or ^{32}P-labelled c'AMP either

for the period before the addition of the gonadotrophin or after the addition of the gonadotrophin. The proteins could be separated by polyacrylamide gel electrophoresis (PAGE), stained, the gels sliced into sections, and the radioactivity counted. To ensure that the differences between incubations are not due to differences in the rate of protein synthesis, and also to investigate whether the phosphorylated or dephosphorylated protein was newly formed: the tissue should be incubated with ^{3}H-Leucine and ^{32}P- and the incorporation of tritium into the protein, whether with or without ^{32}P, could be examined in a similar way to incorporation of ^{32}P alone.

Since we cannot be sure that protein synthesis is the only major event in gonadotrophic stimulation of the ovary, experiments could be designed to investigate the possible role of calcium as a mediator of gonadotrophic hormone action. This ion has been widely implicated as a mediator of hormone action. Janszen et al., (1976) demonstrated that LH-induced testosterone production in rat Leydig cells progressively decreased in the absence of Ca^{++} to one-third of that with Ca^{++} (2.5 mM). However, this decrease was restored (within 30 min) by adding Ca^{++} to the incubation medium, indicating that the integrity of the cells had not been damaged. In the same series of experiments, it was shown that the activation of c'AMP dependent protein kinase by LH/HCG was not affected by the omission of Ca^{++} from the incubation medium, suggesting that Ca^{++} may be involved in steroidogenesis at a stage beyond the LH receptor-adenylate cyclase-protein kinase system (Janszen et al., 1976). In the adrenal gland it has also been demonstrated that the presence of Ca^{++} in the incubation medium is required for the full stimulation of steroidogenesis (Birmingham, Elliot and Valere, 1953;

Sayers, Beall and Seeling, 1972). This investigation could be applied to ovarian cells, maintained in Kreb's Ringer bicarbonate supplemented with glucose and glutamine. One could, for example, examine the effect of Ca^{++} specific ionophores on the steroidogenesis and on the effects of the gonadotrophins. Before more investigations are carried out on the role of Ca^{++} in the regulation of steroidogenesis by trophic hormone, it may be necessary to know more about the 'free' and 'bound' Ca^{++} concentrations in the different cellular compartments under different stimulatory conditions. At this time this knowledge is very sparse.

At this point, it is perhaps appropriate to propose a tentative hypothesis for the mechanism of gonadotrophin-induced steroidogenesis in the ovaries of immature rat primed with PMSG. Knowledge of the biochemical mechanism of the action of trophic hormones on the ovary is limited. This may well reflect the complex nature of the organ with its multiple cell types. However, it has been demonstrated that gonadotrophin-induced steroidogenesis is mediated by protein synthesis. The biochemical sequence from the initial binding of the trophic hormone to its cellular receptors which culminates in the induction or stimulation of steroiodogenesis has not been fully established. A summary of some of the proposed mechanisms in the control of steroidogenesis in the ovary based on what is now known to occur in adrenal cortical cells, are depicted in Fig. 9.2. It is known that the first event is that the hormone binds to a specific receptor on the surface of the cell. This results in the activation of the membrane bound adenylate cyclase, resulting in increased intra-cellular c'AMP levels. c'AMP binds to specific

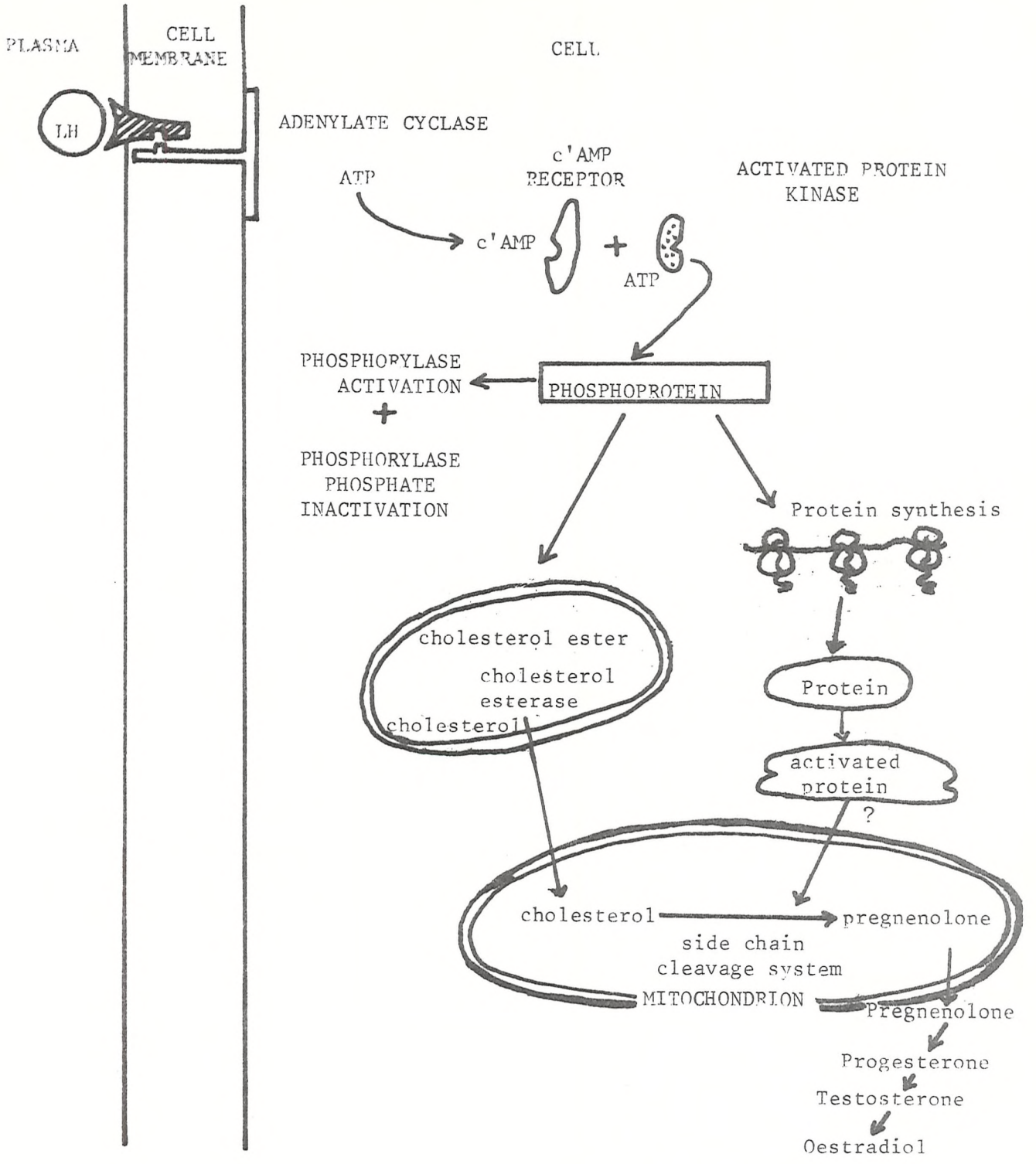


FIGURE 9.2 Proposed model for LH/HCG regulation of steroidogenesis in the ovarian thecal cells. See text for discussion of the various steps shown on the proposed model. (Adapted from Schulster et al., 1976; Marsh, 1976 and Gill, 1979).

intra-cellular receptor protein(s), leading to the activation of a c'AMP dependent protein kinase. It is presumed that stimulation of steroidogenesis is subsequent to the c'AMP dependent phosphorylation mechanisms (Gill, 1979). The phosphorylation mediated activation of phosphorylase and cholesterol esterase and the inactivation of phosphorylase phosphatase and cholesterol ester synthetase result in increased free cholesterol (Schulster et al., 1976; Gill, 1979). Free cholesterol provides the necessary precursor for steroid hormone synthesis (Marsh, 1976) and its supply may indeed be the primary site through which steroid synthesis is regulated. Cholesterol is transported into mitochondria and presented to mitochondrial side chain cleavage enzyme system by cholesterol carrier protein (Gill, 1979). A major consideration in LH/HCG stimulation of steroidogenesis is the requirement for protein synthesis (see Chapter 7). The proposed regulatory protein which is synthesized, is thought to act in the mitochondria between the accumulating free cholesterol and formation of pregnenolone (Schulster et al., 1976). The formation of pregnenolone is rate-limiting in the steroidogenic pathway and hence an increased rate of synthesis results in the increased formation of its many metabolites (Gill, 1979).

In summary, an understanding of the detailed mechanism by which gonadotrophins act on the ovary is lacking, and at this time only potentials, possibilities and some indications of the events in this complex field and for subsequent experimental work can be discussed. The very complexity of this problem immediately suggests caution in making general deductions from a few experiments in one animal species and on relatively few tissues. Data from future studies will decide if we succeed in coming to an understanding of the mechanism of action of trophic hormones on steroidogenesis.

CONCLUSION

1. Antisera raised against oestradiol, testosterone and progesterone have been characterized and found to be highly specific.

2. Maximum ovarian secretion of oestradiol and testosterone have been shown to occur from the ovaries of immature rats primed for 48 h with PMSG, while maximum secretion of progesterone occurred from the 72 h PMSG primed ovaries. This suggests that oestradiol and testosterone are the principal steroids secreted by the preovulatory ovaries, while progesterone is secreted in substantial amounts after ovulation, in immature rat primed with PMSG. A comparable profile of steroid levels in the plasma of the PMSG primed rats was observed and this profile was also similar to that in adult rats during the oestrous cycle.

There was a highly significant correlation between the steroid concentration in the medium and the amount in the tissue. Hence assays on small samples of the medium were routinely used in studies of the steroidogenic response of the ovary to gonadotrophins and inhibitors.

3. The ability of FSH and PMSG to stimulate oestradiol secretion from the granulosa cells of PMSG-primed immature rat ovaries and the ability of LH and HCG to stimulate significant testosterone secretion from Leydig cells of isolated rat testis provides a useful *in vitro* 'tool' for comparing the biological activities of these gonadotrophins.

4. The physiological stimulation of the ovary by gonadotrophins secreted around the time of ovulation seems to have a dual effect: after stimulation, the gonadotrophins inhibit testosterone and oestradiol secretion and stimulates progesterone secretion from the Graafian follicles.

5. 68 h after treatment of 25-day old rats with PMSG, ova were observed in their oviducts. Sodium pentobarbitone injected on the afternoon of the second day after PMSG treatment, delayed the expected ovulation and the expected increase of ovarian progesterone anticipated following endogenous LH surge.

6. It appears that in the whole ovary of the immature rat, primed with PMSG, the supply of testosterone may be rate limiting in the final secretion of oestradiol, since in vitro, oestradiol secretion by these ovaries is enhanced by the addition of testosterone.

7. The enhanced oestradiol secretion (by the PMSG primed ovaries) in the presence of testosterone does not seem to involve the synthesis of protein(s) of short half life since the increased oestradiol secretion in the presence of exogenous testosterone was not significantly affected by cycloheximide, during a 4 h incubation period.

8. Protein synthesis and gonadotrophin-induced progesterone and testosterone secretion are both suppressed by cycloheximide, implicating an involvement of a labile protein in mediating the HCG-induced response. This protein must presumably be involved

at a stage prior to testosterone synthesis.

9. 5α-reduced androgens, which can be found within the ovary of the immature rat, can inhibit oestradiol secretion by granulosa cells isolated from PMSG primed immature rat ovaries. The inhibition of oestradiol secretion by the 5α-reduced androgens was not reduced in the presence of oFSH and/or testosterone, from which it may be concluded that the 5α-reduced androgens have a direct effect on the androgen-aromitizing enzymes. In addition, the 5α-reduced androgens stimulate progesterone secretion from the granulosa cells. The effects of the 5α-reduced androgens have important connotations with respect to the control of the onset of puberty and the regulation of ovarian oestradiol secretion within the micro-environment of an ovarian follicle.

APPENDIX I

EFFECTS OF ACTINOMYCIN D AND TESTOSTERONE ON STEROID SECRETION BY THE OVARY OF THE IMMATURE RAT.

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Fortune & Armstrong (1978) have demonstrated that oestradiol is secreted by the granulosa cells of the preovulatory rat follicle while testosterone is secreted by the theca cells. Lieberman, Barnea, Bauminger, Tsafriri, Collins & Lindner (1975) have shown that macromolecular synthesis is implicated in the action of luteinizing hormone (LH) and human chorionic gonadotrophin (HCG) on the preovulatory rat follicle. These two observations have been used to investigate the interaction between HCG and ovarian testosterone and oestradiol secretion in the immature rat ovary.

Ovaries from 28-day-old pregnant mare serum gonadotrophin (PMSG)-primed (5.0 i.u.s.c. 48 h before death) rats were incubated individually for 4 h in Krebs-Ringer bicarbonate supplemented with 1.1 mM-glucose and 2.0 mM-glutamine, gassed with 5% CO_2/95% O_2, and in the presence of HCG (1 i.u./ml), actinomycin D (8 μg/ml) or testosterone (5×10^{-8} mol/l).

Human chorionic gonadotrophin stimulated secretion of both oestradiol and testosterone; the concentrations in the incubation medium at the end of the 4 h period were $12.8 \times 10^{-11} \pm 2.1 \times 10^{-11}$ (S.E.M.) mol/l and $38.3 \times 10^{-11} \pm 4.5 \times 10^{-11}$ mol/l in control medium and HCG-stimulated culture dishes respectively for oestradiol ($P < 0.02$) and $21.1 \times 10^{-10} \pm 7.0 \times 10^{-10}$ mol/l and $52.4 \times 10^{-10} \pm 24.3 \times 10^{-10}$ mol/l for testosterone ($P < 0.05$).

The addition of testosterone to the medium significantly enhanced oestradiol secretion, from $24.8 \times 10^{-11} \pm 4.7 \times 10^{-11}$ mol/l to 106.3×10^{-11} mol/l ($P < 0.001$) in control and experimental dishes respectively.

Actinomycin D blocked the HCG-stimulated secretion of both oestradiol and testosterone, the levels of oestradiol being reduced from $38.3 \times 10^{-11} \pm 4.5 \times 10^{-11}$ mol/l to $27.6 \times 10^{-11} \pm 4.8 \times 10^{-11}$ mol/l, and of testosterone from $52.4 \times 10^{-10} \pm 24.4 \times 10^{-10}$ mol/l to $32.0 \times 10^{-10} \pm 19.8 \times 10^{-10}$ mol/l ($P < 0.02$ and < 0.05 respectively). Addition of testosterone in the presence/

presence of actinomycin D and HCG partially reversed the inhibition of oestradiol secretion, but at the concentrations of HCG and testosterone used here, the reversal was not significant.

The results are consistent with the hypothesis that the oestradiol, secreted in response to HCG by the ovary of the PMSG-primed rat, is due primarily to an increased testosterone production, which acts as substrate for aromatization.

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APPENDIX II

INHIBITION OF OESTRADIOL SECRETION FROM ISOLATED GRANULOSA CELLS BY 5 α-REDUCED ANDROGENS.

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It is now well established that aromatisation of androgens in the ovary of the immature rat occurs predominantly in the granulosa cell layer, and that it is stimulated by FSH (Dorrington, Moon and Armstrong, 1975). However, the processes which control this activity are not well understood. Recently Hillier, van der Boogaard, Reichert and van Hall (1980) have demonstrated that the 5 α-reduced androgens, dihydrotestosterone and androstanedione, inhibited aromatisation of testosterone by granulosa cells of the rat, while McNatty, Makris, de Grazia, Osathanondh and Ryan (1979) reported that there were high levels of the 5 α-reduced androgen, dihydrotestosterone, in follicular fluid of human ovaries. However, the 5 α-reduced androgens 3β17β-androstanediol and 3α17β-androstanediol are major ovarian metabolites in the immature rat (Eckstein, Mechoulam and Burstein, 1970). We have investigated the effects of these steroids on oestradiol secretion by isolated granulosa cells of the rat.

Granulosa cells were isolated from pre-ovulatory follicles of immature rats which had been primed with 5.0 i.u. PMSG on day 26. The cells were suspended in Kreb's Ringer Bicarbonate buffer supplemented with glucose and glutamine, and incubated at 37°C for four hours. Testosterone (1×10^{-7}M) ovine FSH (0.1 μg/ml medium), and one of the 5α-reduced androgens (1×10^{-6}M) were added to the medium before incubation. The 5α-reduced androgens used were dihydrotestosterone, androstanedione and 3β17β-androstanediol and 3α17β-androstanediol. The incubation medium was subsequently assayed for oestradiol by a specific radioimmunoassay.

Testosterone enhanced oestradiol secretion from these cells, from 5.76 ± 0.22 pmole/10^5 cells/4 hr, to 24.12 ± 1.60 pmole/10^5 cells/4 hr ($P < 0.001$). The secretion of oestradiol was further enhanced by the addition of ovine FSH, to 24.97 ± 1.8 pmole/10^5 cells/4 hr. The addition of dihydrotestosterone in the presence of testosterone and FSH suppressed the oestradiol secretion to 13.04 ± 0.75 pmole/10^5 cells/4 hr ($P < 0.001$, compared with the cells incubated with testosterone and FSH), representing 52.2% of the previous aromatising activity. Secretion of oestradiol was also/

also suppressed from the levels caused by the addition of both FSH and testosterone by androstanedione (50.7%) by 3α17β-androstanediol (16.4%) and by 3β17β-androstanediol (24.4%).

These results confirm previous reports that androstanedione inhibits oestradiol secretion from granulosa cells of pre-ovulatory follicles of the rat, and also demonstrate that other major 5α-reduced androgens found in the ovary of the immature rat cause a potent suppression of aromatisation of exogenous testosterone.

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